The Journal of Biochemistry, published monthly by
the Japanese Biochemical Society, Tokyo, Japan.
Vol. 44, No. 4, April 25, 1957

Vol. 44, No. 4 **April, 1957**

THE JOURNAL OF BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

EDITORIAL BOARD

SHIRO AKABORI **SHIGERU AKAMATSU**
SHICHIRO AKIYA **FUJIO EGAMI**
SABURO FUNAHASHI **YOSOJI ITO**
KOOZOO KAZIRO **KATASHI MAKINO**
TOMOO MIWA **ZIRO NIKUNI**
NORIO SHIMAZONO **TARO TACHIBANA**
YUSUKE SUMIKI **HIROSHI TAMIYA**
HARUHISA YOSHIKAWA

PUBLISHED MONTHLY
BY
THE JAPANESE BIOCHEMICAL SOCIETY
Tokyo University, Tokyo

AGENCIES

Charles E. Tuttle
Rutlant, Vt.
U.S.A.

Maruzen Co. Ltd.
Tokyo,
Japan

Santo Vanasio
Köln
Germany

PRINTED IN JAPAN

THE JOURNAL OF BIOCHEMISTRY was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

This JOURNAL is collecting the original investigations on the biochemical subject. It is published monthly, one volume per year.

Note to Contributors

All manuscripts should be carefully typewritten, triple-spaced, in English, French or German, and addressed to *The Editor, The Journal of Biochemistry, The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Authors receive 50 reprints of their papers as a rule at cost ; additional copies can be prepared by the order on cost accounts. All orders for reprints should be clearly noted on the article paper or in a sheet of paper attached to it. On submitting the paper, it is preferable to attach the non-traced charts besides original drawings, for circulation to Editors.

On request from authors to contribute to JOURNAL, the *Directions for preparation of manuscripts*, including styles of abbreviations of symbols, terms, units and periodicals, will be sent immediately.

Subscription

Subscription Price. ¥3,600 (U. S. $10.00) per volume, postage inclusive. Back issues are available for sale, from volume 1 up-to-date at ¥3,600 (U. S. $10.00) per volume, postage inclusive.

Remittances should be made preferably by check or draft payable to *The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Foreign subscription is treated by the agencies of *Charles E. Tuttle, Co.*, Rutland, Vermont, U.S.A., *Santo Vanasio*, Köln, Germany, and *Maruzen Co. Ltd.*, Chuo-ku, Tokyo, Japan.

The Journal of Biochemistry, Vol. 44, No. 4, 1957

PHYSIOLOGICAL CHEMISTRY OF THE HARD TISSUES

II. UPTAKE OF RADIOACTIVE CALCIUM FROM THE DIET BY INCISOR TOOTH AND BONE OF RAT

By YOSOJI ITO, SUSUMU TSURUFUJI, AND EICHI MURAI

(From the Institute of Physiological Chemistry, Department of Pharmacy, Faculty of Medicine, University of Tokyo, Tokyo)

(Received for publication, November 28, 1956)

Radioactive calcium and inorganic phosphate may enter skeletal tissues with ease by physicochemical processes (*1*). These processes may, therefore, interfere in the examination of calcification of skeletal tissues by isotopic tracers (*2*, *3*). In a previous study (*4*), the authors estimated the ratio of radiocalcium uptake by physicochemical exchange to that due to physiological deposition resulting from new tissue formation in continuously growing incisor tooth of a rat. About sixty per cent of the total incorporation of radiocalcium was found to be due to physiological accretion of newly deposited mineral and the remaining forty per cent is attributable to physicochemical exchange in the initial stage of radiocalcium incorporation. On the other hand, quantitative estimation of radiocalcium or radiophosphate uptake in skeletal tissues by physicochemical processes has not yet been established.

The present study proposes that there is some difference in the mechanism of radiocalcium incorporation between continuously growing incisor and skeleton system in general. In addition, a conventional balance experiment was carried out to evaluate the degree of correlation among growth, food consumption, and intake, excretion, and retention of calcium by comparing correlation coefficients.

EXPERIMENTAL AND RESULTS

Groups of litter mates of growing male albino rats were used in this experiment.

Each of the rats was placed separately in a metabolism cage and was given an adequate mixed diet and distilled water *ad libitum*.

Composition of the diet is:

Wheat flour	70
Purified casein	20
Brewer's yeast	5
Vegetable oil	5
$CaCO_3$	1.25
KCl	1.1
NaCl	0.669
$MgSO_4 \cdot 7H_2O$	0.406
$MnSO_4 \cdot 4H_2O$	0.0200
$FeSO_4 \cdot 7H_2O$	0.0943
KI	0.0103
$ZnSO_4 \cdot 7H_2O$	0.0021
$CuSO_4 \cdot 5H_2O$	0.0012
Distilled water	60

After calcinating the diet its calcium content was determined gravimetrically by precipitating as calcium oxalate and calcium was found to be 0.322 per cent.

The phosphorus content, calculated from a table of nutritive values issued by the National Institute of Nutrition (*5*), was 0.294 per cent. From these values the Ca/P ratio of the diet was calculated as 1.1, which is quite suitable for the nutritive requirement of the two elements. After mixing all components of the diet, the resultant wet mash was put in a glass feeding pot, and heated in a steamer to make up steamed cake rations. Construction of the cage and form of the rations were suitable for the balance experiment so that neither scattering of the food by the animals nor contamination of excreta with dietary scraps were observed.

Moreover, this steamed cake ration is quite suitable for preparing a diet containing radioactive calcium. A calcium deficient diet may be prepared by removing calcium carbonate from the diet. Therefore, addition of calcium carbonate labeled with Ca^{45} to the diet in place of non-labeled calcium gives a diet containing uniformly labeled calcium. This is suitable for the tracer experiment on the metabolism of dietary calcium. The labeled diet was prepared as follows: all components of the diet other than calcium carbonate were mixed and were made into wet mash. On the one hand, labeled calcium carbonate was precipitated from a solution of calcium chloride containing Ca^{45}. After centrifugation, precipitated calcium carbonate was suspended in a little water and added to the wet mash. After complete mixing, it was made up into the steamed cake rations as described above. All the procedures of handling radioactive substance were carried out under wet conditions

TABLE I

Growing Rate, Intake of Diet, Calcium Balance of Rats, and Measurements of Their Femoral Bone and Lower Incisor Tooth

Rats were placed separately in metabolism cages and given a suitable synthetic diet throughout the experimental period.

Litter No.		I	II	III	IV	Mean***
Age in days at the beginning of experiment		39	39	38	36	
No. of animals		8	3	6	3	
Intake of diet	Full term	574 *g.*	521	500	464	515
	Day 1– 8*	22.1	21.0	19.6	17.2	20.0
	Day 9–13*	26.5	22.4	22.2	22.8	23.5
	Day14–18*	28.4	25.2	23.9	22.6	25.6
	Day19–22*	30.7	28.6	28.3	24.7	28.1
Gain in body weight	Full term	132 *g.*	113	108	102	114
	Day 1– 8*	6.3	5.5	5.2	5.2	5.6
	Day 9–13*	6.0	4.9	4.3	4.8	5.0
	Day14–18*	5.1	4.2	4.2	3.8	4.3
	Day19–22*	4.6	5.0	4.8	3.8	4.6
Ca intake	Full term	1848 *mg.*	1676	1611	1494	1657
	Day 1– 8*	71.1	67.7	63.0	55.5	64.3
	Day 9–13*	85.2	72.1	71.6	73.5	75.6
	Day14–18*	91.6	81.1	77.0	72.8	80.6
	Day19–22*	98.9	92.0	91.1	79.7	90.4
Ca excretion	Full term	1225 *mg.*	1123	1051	941	1085
	Day 1– 8*	54.0	52.5	50.3	45.0	50.5
	Day 9–13*	44.8	41.0	35.3	36.6	39.4
	Day14–18*	60.9	52.7	50.7	42.9	51.8
	Day19–22*	65.9	58.5	54.8	46.0	56.3
Ca retention	Full term	623 *mg.*	553	560	553	572
	Day 1– 8*	17.1	15.2	12.7	10.5	13.9
	Day 9–13*	40.4	31.1	36.3	36.9	36.2
			(31.6)**	(37.2)**		(36.5)**
	Day14–18*	30.7	28.4	26.3	29.9	28.8
	Day19–22*	33.0	33.5	36.3	33.7	34.1
Femur (bilateral)	Wet weight	978 *mg.*	929	897	921	931
	dried weight	508	472	465	459	476
	ash weight	275	258	254	247	259
	amount of Ca	105	99	97	95	99
Lower incisor (bilateral)	Dried weight	96.5 *mg.*	96.0	97.0	99.7	97.3
	amount of Ca	27.5	27.0	28.8	27.9	27.8

* Average daily amount per rat.

** Terms in parentheses are the values corresponding to those in Table II. See also notation ** in Table II.

*** Simple mean.

so that dangerous radioactive dust contamination could be avoided.

After preliminary feeding of about one week on the diet, the balance experiment was brought into operation. The radioactive diet containing labeled calcium carbonate was given on the 9th, 10th, and 11th days of the experiment.

Ingestion of the rations, excretion of calcium, and gain in body weight were measured at intervals and the results are summarized in Table I.

Intake of calcium was calculated from amount of the rations taken and calcium content of the diet. Retention of calcium was calculated by subtracting the amount excreted from that ingested.

At the end of the 22nd day of the experiment, all the rats were sacrificed by cutting the carotid artery. Their lower incisors and femurs were removed without adherent soft tissues and raw weight, dry weight dehydrated at 100° to 105°, ash weight, and calcium content were measured, which are tabulated in Table I.

The total amounts of labeled calcium ingested on the 9th, 10th, and 11th days of the experiment are shown in Table II. Excretion and retention of labeled calcium on the 13th, 18th, and 22nd days of the experiment and the amount of dietary labeled calcium found in the femurs and lower incisors at the end of the experiment are also shown in Table II. Retention was calculated by subtracting the amount of excreted radiocalcium from that ingested. All the activity assays of Ca^{45} and quantitative analyses of calcium were carried out as $Ca(COO)_2 \cdot H_2O$, as described in the previous report (*4*), with the exception of using glass Petrie dishes (diameter, 26±0.5 mm.) in place of porcelain crucibles as sampling dishes. All radioactivity assays were carried out by means of a thin mica window G-M counter and were corrected for radioactive decay, self absorption, sample geometry, and for changes in efficiency of the counter.

DISCUSSION

Statistical analysis of original data has shown that there are significant differences among groups of litter mates in growth, consumption of food, and ingestion and excretion of calcium. Other experiments in this laboratory have often shown that there were some statistically significant differences among litters. Therefore, in the design of an experiment for statistical analysis, the effect of litters should be treated as one of the important sources of potential variations.

In the balance experiment (Table I) it is shown that average daily net increase of body calcium of the rat was 36.5 mg. during the period from the 9th to the 13th day of the experiment. Since more than 99 per cent of total body calcium of a rat is contained in the skeleton (*6*), it may be concluded that the total amount of retained calcium during the experimental period should be accounted for by the skeletal growth. Because skeletal calcium originates entirely from the diet, the increase in skeletal calcium must be supplied from the diet from day to day. Therefore, when a diet containing uniformly labeled calcium was ad-

Table II

Administration of Diet Containing Uniformely Labeled Calcium into Rats in the Course of Calcium Balance Experiment

Diet containing labeled Ca was given on 9–11th day in the course of calcium balance experiment. Ingestion, excretion and retention of labeled calcium and its incorporation into femoral bone and lower incisor were measured.

Litter No.		I	II	III	IV	Mean***
No. of animals		8	2**	5**	3	
Total labeled Ca ingested on the 9th, 10th, and 11th days of expt.		253 *mg.*	235	220	227	234
Labeled Ca excretion	Total (Day 9–22)	91 *mg.*	100	84	81	89
	Day 9–13*	16.4	18.0	14.6	14.0	15.7
	Day14–18*	1.2	1.5	1.6	1.7	1.4
	Day19–22*	0.5	0.5	0.5	0.6	0.5
Labeled Ca retention	On Day 13	171 *mg.*	145	148	157	155
	On Day 18	164	138	139	149	148
	On Day 22	162	135	136	149	146
Dietary labeled Ca found in	Femur	5.76 *mg.*	5.22	4.90	4.87	5.19
	Lower incisor	1.35	1.28	1.25	1.32	1.30

* Average daily excretion of labeled Ca per rat.

** Owing to missing values, numbers of rats are reduced in litters II and III in this Table as compared with Table I.

*** Simple mean.

ministered to the rat, 36.5 mg. of labeled dietary calcium should be retained per day to sustain the skeletal growth, and on the other hand, not more than 36.5 mg. would have had to be retained. Actually, however, a total of 155 mg. of labeled dietary calcium had been stored at the end of the 13th day of the experiment by the rat given a diet containing radioactive calcium for three consecutive days from the 9th to the 11th day of the experiment. In other words, at least 155/3 or 51.7 mg. of labeled calcium from the diet had been stored daily by the rat. There is some difference between these two determinations, *i.e.* 51.7−36.5=15.2 mg., which is almost one-half of the daily retention. Such a discrepancy may be accounted for mainly by physicochemical exchange of Ca^{45} ions in body fluid with Ca^{40} of bone crystals (*7*).

In bone there are more complex processes, including not only physicochemical exchange but also physiological reconstruction processes of the bone. Physiological reconstruction would also play any important role in the mechanism of depositing excess radioactive calcium in the bone than would be expected from skeletal growth.

On the other hand, it is shown in Table II that a lower incisor of the rat kept 1.30 mg. of dietary labeled calcium at the end of the experiment. As explained above, since Ca^{45} in the incisor originated from the labeled dietary calcium administered during the three consecutive days, it is induced that the amount of labeled calcium resulting from a one day's diet is 1.30/3 or 0.433 mg. As reported in the previous work (*4*), a continuously growing lower incisor tooth of a rat takes up about 0.62 mg. of calcium daily. This depositing calcium must be supplied from the daily diet. Therefore, 0.433 mg. of labeled dietary calcium found in the lower incisor in this experiment may be significantly less than the amount of calcium deposited daily in the incisor.

According to an autoradiographic study in this laboratory (unpublished), Ca^{45} that has once entered the animal body continues to be deposited in the incisor for a long time, though in rather a small amount, after its immediate large deposition.

Since 0.433 mg. of labeled calcium was found on the 22nd day, it may be concluded that less than 0.433 mg. per day of labeled dietary calcium would have been found in the incisor on the 13th day of the experiment. In other words, therefore, at least 0.2 mg. of calcium had remained to be deposited later. There is a remarkable difference between bone and incisor in the uptake of dietary labeled calcium. Physicochemical exchange and physiological reconstruction processes predominate in the fixation of labeled calcium in the bone, while physiological accretion occurs predominantly in the rapidly growing incisor tooth of a rat. From the original data, sample correlation coefficients between gain in body weight and consumption of food, intake of calcium and its excretion, and intake of Ca^{45} and its incorporation into the incisor or femoral bone, etc. were calculated and are summarized in Table III.

TABLE III

Correlations among Various Determinations in Rats in Balance Experiment

r : Sample correlation coefficient, p : Probability of t calculated for testing the significance or r (*8*), ρ : 99 per cent fiducial intervals of correlation coefficient in population.

Correlation coefficient between		r	p	ρ
Uptake of diet and body weight gain	Full term	0.934	<0.01	$0.775<\rho<0.979$
	Day 1– 8	0.754	<0.01	$0.342<\rho<0.922$
	Day 9–13	0.899	<0.01	$0.687<\rho<0.970$
	Day 14–18	0.813	<0.01	$0.477<\rho<0.946$
	Day 19–22	0.259	—	—
Uptake and excretion of Ca	Full term	0.918	<0.01	$0.740<\rho<0.976$
	Day 1– 8	0.612	<0.01	$0.056<\rho<0.878$
	Day 9–13	0.738	<0.01	$0.380<\rho<0.973$
	Day 14–18	0.862	<0.01	$0.570<\rho<0.962$
	Day 19–22	0.923	<0.01	$0.737<\rho<0.979$
Uptake and retention of Ca	Full term	0.552	<0.05	$-0.004<\rho<0.847$
	Day 1– 8	0.810	<0.01	$0{\cdot}441<\rho<0.945$
	Day 9–13	0.687	<0.01	$0.213<\rho<0.899$
	Day 14–18	0.362	—	—
	Day 19–22	0.060	—	—
Uptake and retention of Ca^{45}	Day 9–13	0.837	<0.01	$0.497<\rho<0.954$
	Day 9–22	0.811	<0.01	$0.443<\rho<0.945$
Ca^{45} Retention and its uptake by	Femur	0.404	—	—
	incisor	0.803	0.01	$0.416<\rho<0.944$
Femnr Ca^{45} and incisor Ca^{45}		0.254	—	—

From the viewpoint of correlation it is concluded that the more an animal takes food, the more the body weight increases, as expected generally. On the contrary, correlation coefficient between intake and retention of calcium is rather small, and correlation coefficient between intake and excretion of calcium is significantly larger than the former (at 1 per cent level of significance). As the intake of calcium was proportional to the amount of food taken in this experiment, it is suggested that rats which took more food (and consequently calcium also) grew better, but their skeletal growth was not closely dependent upon their calcium intake (food intake either) itself, larger intake of calcium causing larger excretion of calcium than its retention. It is of some interest that the degree of correlation between gain in body weight and consumption of food, and that between intake of calcium and its retention gradually decreased with the passage of days.

There is a remarkable difference between incisor and femur in correlations of their Ca^{45} uptakes to total retention of dietary Ca^{45}, and consequently no correlation is observed between femur and incisor in their Ca^{45} uptake. The fact also suggests that the mechanism of incorporation of Ca^{45} into the incisor of a rat is different from that in the growing femoral bone.

SUMMARY

Groups of litter mates of growing male albino rats were placed on an adequate synthetic diet and a calcium balance experiment was carried out, not only by conventional means, but also with the aid of radioactive calcium. After sacrifice of the rats, their lower incisor teeth and femoral bones were removed and analysed.

Correlation coefficients among food intake, gain in body weight and intake, excretion and retention of ordinary and radioactive calcium were calculated.

Conclusions drawn from this experiment are as follows:

1. Bony skeleton of growing rats takes more radioactive calcium from the diet than expected from the increase of skeletal calcium by bone growth, while continuously growing incisor tooth takes less Ca^{45} than expected from the rate of incisor growth.

These facts may indicate a difference in the mechanism of radioactive calcium incorporation between bony skeleton and continuously growing incisor in a growing rat.

Radioactive calcium may enter the skeletal tissue by at least two principal processes, physiological tissue formation and physicochemical exchange. On the contrary, it is supposed that incisor tooth of the rat which is growing actively receives radioactive calcium mainly by physiological tissue formation.

2. While the rate of growth of animals is parallel with their food ingestion, skeletal growth is not so closely related to calcium intake

(nor to food intake) as body growth. A larger intake of calcium causes instead a larger excretion of calcium than retention.

3. Little correlation between incisor and femur in the uptake of radioactive calcium suggests a difference in the mode of radiocalcium uptake.

REFERENCES

(*1*) Neuman, W. F., and Neuman, M. W., *Chem. Revs.*, **53**, 1 (1953)

(*2*) Sachs, J., Radioactive tracers in biochemistry and physiology, McGraw Hill, New York, p. 284, 1953

(*3*) Neuman, W. F., and Riley, R. F., *J. Biol. Chem.*, **168**, 545 (1947)

(*4*) Ito, Y., Tsurufuji, S., Hiramatsu, Y., and Chiba, M., *J. Biochem.*, **43**, 701 (1956)

(*5*) The National Institute of Nutrition of Japan (ed.), Handbook of nutritive values of various food materials (in Japanese), Daiichi Publ. Co., Tokyo, (1953)

(*6*) Mitchell, H. M., Hamilton, T. S., Steggerda, F. R., and Bean, H. W., *J. Biol. Chem.*, **158**, 625 (1945)

(*7*) Harrison, H. E., and Harrison, H. C., *J. Biol. Chem.*, **185**, 857 (1950)

(*8*) Goulden, C. H., Methods of statistical analysis, John Wiley and Sons, New York, p. 72, (1939)

The Journal of Biochemistry, Vol. 44, No. 4, 1957

STUDIES ON EGG YOLK PROTEINS

II. ELECTROPHORETIC STUDIES ON PHOSVITIN, LIPOVITELLIN, AND LIPOVITELLENIN

By HIROSHI SUGANO

(*From the Department of Chemistry, Faculty of Science, Niigata University, Niigata*)

(Received for publication, December 17, 1956)

It has been generally accepted that the yolk protein consists of two main fractions, lipovitellin, the water-insoluble part, and livetin, the water-soluble fraction. The original method of preparing the lipovitellin which derived from the work of Osborne and Campbell (*1*) was somewhat modified by Chargaff (*2*). Recently, Alderton and Fevold (*3*) reported a different method for isolating lipovitellin, and, also, Fevold and Lausten (*4*) isolated another lipoprotein which was termed lipovitellenin by them. The results of these workers gave an evidence that the former lipovitellin is not homogeneous. However, up to the present, no information has been given for the homogeneity of these preparations. Consequently, some obscurity and confusion exist in the use of the term of lipovitellin. Mecham and Olcott (*5*) have isolated a phosphorus-rich protein, phosvitin, from the yolk by precipitating as the magnesium salt. The presence of phosvitin in the lipovitellin of Alderton and Fevold has been indicated by its isolation from this preparation by Mecham and Olcott (*5, 6*). However, the correlations of phosvitin with the above isolated lipoproteins must be made more clear in order to give a further insight to the protein composition of the yolk.

In a previous work, the author has found the suitable condition for the electrophoretic study of yolk proteins and showed the electrophoretic patterns of the total yolk protein and its several fractions obtained by water dialysis (*7*). In the present work, the author has undertaken the electrophoretic studies concerning the lipovitellin of Alderton and Fevold, and the lipovitellenin of Fevold and Lausten, and the isolation process of the phosvitin. Experiments have given some evidences for the inhomogeneity of the former two preparations and the correlative relationships between these preparations and the phosvitin. For representing the protein compositions of these preparations, the author has provisionally named " α-lipovitellin " and " β-lipovitellin " for two main components of the yolk protein, both of which have shown a single peak under the electrophoretic condition used.

EXPERIMENTAL

Materials—The fresh eggs of White Leghorn were obtained by the courtesy of the Poultry Department of Faculty of Agriculture or purchased at retail market. The yolks were washed in tapwater and rolled on filter paper to free them from adhering white. The yolk membrans were then punctured and the contents allowed to drain out.

The lipovitellin was prepared according to the method of Alderton and Fevold (*3*). The centrifugal separation was carried out in Spinco Model L instrument. The precipitate was washed once by water in the centrifuge. For the estimation of lipid and phosphorus, the precipitate was suspended in a small amount of water, extracted with ether to remove the uncombined lipid, and after centrifugation the precipitate was dried in a high vacuum. For the purpose of electrophoretic analysis, it was dissolved in 10 per cent sodium chloride solution, extracted with ether, and then dialyzed against buffer solution.

The lipovitellenin was prepared by the method of Fevold and Lausten (*4*). For the estimation of lipid and phosphorus, the precipitate was allowed to dry in a high vacuum. In the electrophoretic analysis, it was dissolved in 10 per cent sodium chloride solution by stirring with ether, or dispersed in water and dissolved easily by the careful addition of 0.1 *N* sodium hydroxide. The transparent solution could be obtained at pH 8.5 to 9.0.

The phosvitin was prepared according to the method of Mecham and Olcott (*5*). For the estimation of phosphorus, the phosvitin fraction, which was obtained by the saturation of ammonium sulfate at pH 4.0, was dialyzed against water and then dried by lyophilization.

Analytical Method—The total phosphorus was determined by the method of Allen (*8*). In order to determine moisture content, the sample was dried to constant weight at 65° *in vacuo* over phosphorus pentoxide (Abderhalden pistol heated with methanol).

The total lipid was determined as follows : About 300 to 400 mg. of the sample was put in the extraction tube (1.5 cm. in diameter and 7.0 cm. in height) and dried to constant weight in the Abderhalden pistol and weighed. The material was extracted with 10 ml. of 95 per cent ethanol by shaking for 15 minutes and then centrifuged for 15 minutes at 3,000 r.p.m. After this procedure was repeated 15 times, the material was allowed to dry again to constant weight and the loss in weight was determined. The lipid and phosphorus contents were all estimated on dry basis.

Electrophoresis—Electrophoresis was carried out in a Tiselius apparatus with a cylindrical lens-diagonal slit optical system. The buffer solution which consisted of sodium carbonate-bicarbonate was used at pH 9.8 and ionic strength 0.15 (0.05 depended on sodium chloride). The pH was measured with Beckman Model G instrument at a temperature near 0°. The protein solution was dialyzed for 2 to 3 days before electrophoresis by using a cellophane bag. The protein concentration was determined refractometrically. The specific conductivity of the dialyzed protein solution was measured at 0°. The mobility was calculated from the boundary velocity in the descending limb of the cell. The boundary velocity was estimated from the distance between the protein and epsilon boundaries. The area of boundary was calculated

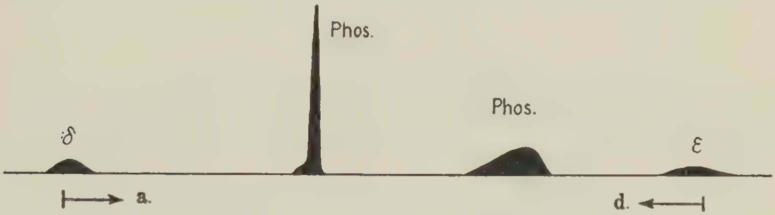


Fig. 1. Electrophoretic pattern of phosvitin prepared by the method of Mecham and Olcott; at pH 9.8 and ionic strength 0.15, after 120 minutes at a potential gradient of 3.6 volts/cm.

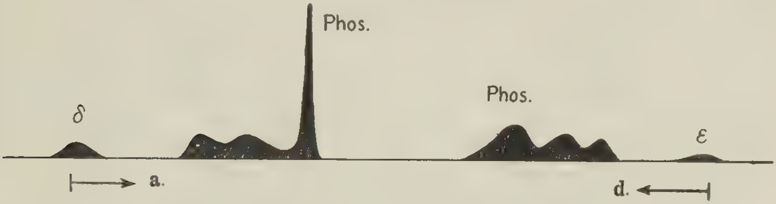


Fig. 2. Electrophoretic pattern of the fraction precipitated at the concentration of 0.09 *M* magnesium sulfate in the method of Mecham and Olcott; at pH 9.8 and ionic strength 0.15, after 120 minutes at a potential gradient of 3.5 volts/cm.

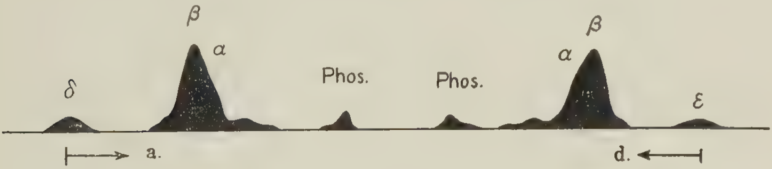


Fig. 3. Electrohporetic pattern of the total yolk protein after ether extraction; at pH 9.8 and ionic strength 0.15, after 180 minutes at a potential gradient of 3.2 volts/cm.

by the weighing method and the values of ascending and descending boundaries were averaged. It was presumed that the refractive index increments of various protein components are all identical.

RESULTS AND DISCUSSION

Phosvitin and Its Isolation Process

An electrophoretic pattern of isolated phosvitin is shown in Fig. 1. A small component could be recognized at the back skirt of the main component. It was already reported by Mecham and Olcott (*5*) that two components were distinguished in the pattern of phosvitin and these two components were separated better in a citrate buffer than in an acetate buffer at pH 4.6 and ionic strength 0.1. The pattern obtained in the present work was very similar to the one obtained by using the acetate buffer. The ascending and descending mobilities of the main component of phosvitin were -14.6 and -11.1 units[1], respectively. This remarkable asymmetry in the mobility, as well as in the boundary shape, dues probably to the high charge density on phosvitin molecule. When a very small amount of phosvitin was involved in another fraction, such asymmetry could not be observed and the two mobilities were -11.7 and -11.3 units, respectively.

An electrophoretic pattern of the 0.09 *M* magnesium sulfate precipitate, which was obtained at the first step of preparing the phosvitin by the method of Mecham and Olcott (*5*), is shown in Fig. 2. The clear solution for electrophoresis was obtained as follows: The soft sticky precipitate collected by centrifugation, was dissolved in 10 per cent sodium chloride solution, extracted with ether thoroughly, and dialyzed against buffer solution. Three main components could be recognized in the pattern. The fastest-moving component was identical with the phosvitin and corresponded to 40 to 44 per cent of the precipitate (Table II). By comparing Fig. 1 with Fig. 2, it was evident that the two slow-moving components were removed perfectly by the subsequent purifying procedure. An electrophoretic analysis of the supernatant solution of the 0.09 *M* precipitate showed that it contained hardly any phosvitin component. Therefore, from the percentages of phosvitin in the total yolk protein and 0.09 *M* precipitate, it could be calculated that about 20 per cent of the former was precipitated as the latter. This value is nearly close to the one (23 per cent) reported by Mecham and

---

[1] $= 10^{-5}$ cm.^{2} volt^{-1} second^{-1}.

Olcott (*5*).

An electrophoretic pattern of the total yolk protein after ether extraction is shown in Fig. 3 (*7*). The phosvitin represented an average of 8.5 per cent of the total yolk protein. If the differences in the material and the analytical method are considered, it would be thought that this value is in quite agreement with the one (6.5 to 7.0 per cent) reported by Mecham and Olcott (*5*).

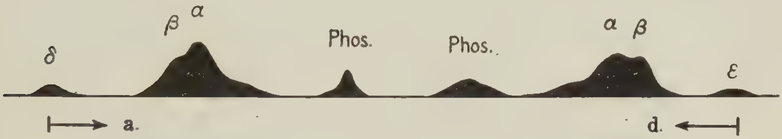


FIG. 4. Electrophoretic pattern of the lipovitellin fraction prepared by the method of Alderton and Fevold; at pH 9.8 and ionic strength 0.15, after 180 minutes at a potential gradient of 3.6 volts/cm.

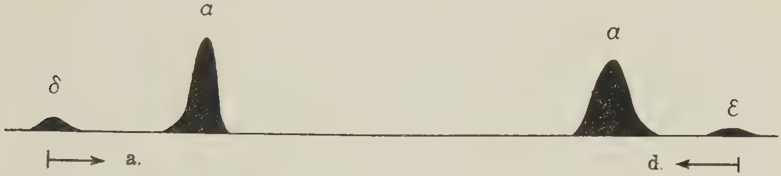


FIG. 5. Electrophoretic pattern of α-lipovitellin fraction; at pH 9.8 and ionic strength 0.15, after 210 minutes at a potential gradient of 3.1 volts/cm.

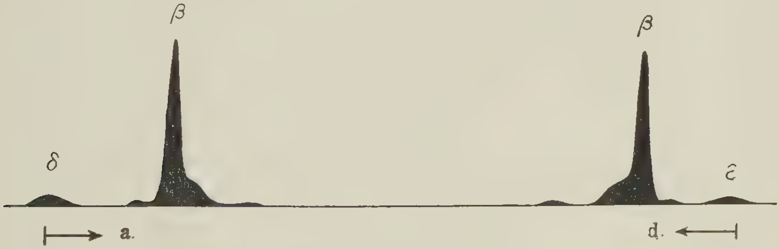


FIG. 6. Electrophoretic pattern of the lipovitellenin fraction prepared by the method of Fevold and Lausten; at pH 9.8 and ionic strength 0.15, after 210 minutes at a potential gradient of 3.1 volts/cm.

The lipid and phosphorus contents of the isolated phosvitin is given in Table I. Both the authors data and those of Mecham and Olcott (*5*) have fairly agreed.

The isolated phosvitin indicated an albumin-like solubility ; namely, the phosvitin has completed to precipitate from aqueous solution only by the saturation of ammonium sulfate at the pH near 4, and it has not been able to make it precipitate from salt solution by removing electrolyte by water dialysis, and also the lyophilized phosvitin has dissolved easily in water. When the phosvitin coexists with other yolk proteins, it can be precipitated easily by dialysis. This is probably because the phosvitin has a high affinity to the other proteins at neutral pH presumably through electrostatic bonds (*5*).

Lipovitellin Preparation of Alderton and Fevold

An electrophoretic pattern of the lipovitellin prepared by the method of Alderton and Fevold (*3*) is shown in Fig. 4. It is apparent from the pattern that this preparation is a complete mixture. And also, it has been found from the pattern of the supernatant solution of the lipovitellin that the total phosvitin in the yolk was precipitated in the lipovitellin preparation by means of centrifugation of the diluted yolk for 60 minutes at 40,000 r.p.m. ; whereas, at 3,000 r.p.m., a very small amount of phosvitin remained in the supernatant even by centrifugation for 90 minutes. Therefore, from the percentages of phosvitin in the lipovitellin and total yolk protein, it is possible to estimate that about 50 per cent of the latter precipitates as the former by the method of Alderton and Fevold. The protein composition of this preparation is given in Table II. Thus, the lipovitellin preparation is such a perfect mixture that there exists no overwhelming component, but α-lipovitellin is a somewhat superior component. The α-lipovitellin had a mobility of —5.3 units and could be isolated easily in almost pure state by the method stated in the next section. The β-lipovitellin had a mobility of —4.4 units and, as described in the latter section, it is a main constituent of the lipovitellenin of Fevold and Lausten. The " other components " represents all residual components except phosvitin, α-, β-lipovitellin, and livetin fraction. The presence of phosvitin in the lipovitellin of Alderton and Fevold was already indicated by its isolation from this preparation by Mecham and Olcott (*5*, *6*). This fact has been proved here quantitatively from the result of electrophoretic analysis of the lipovitellin.

The lipid and phosphorus contents of the lipovitellin is given in Table I, comparing with the values reported in the original (*3*). From the phosphorus contents before and after alcohol extraction, it could be calculated that about 72 per cent of the total phosphorus and about 83 per cent of the protein phosphorus of the lipovitellin are attributed to the 16 to 18 per cent phosvitin which was involved in it. And similarly, about 81 per cent of the lipid of the lipovitellin was accounted for the lipid of β-lipovitellin which was contained 26 to 29 per cent in it2).

TABLE I

*Lipid and Phosphorus Contents of Lipovitellin, Lipovitellenin, and Phosvitin**

	Lipovitellin	Vitellin	Lipovitellenin	Vitellenin	Phosvitin	References
	%					
Lipid	13–14	—	36–38	—	≒0	The Author
	16–18	—	36–41	—	≒0	(*3, 4, 5*)
Phosphorus	2.2–2.4	1.9–2.1	1.6–1.7	0.25–0.3	9.7	The Author
	2.3–2.4	2.0–2.2	1.6–1.8	0.25–0.3	9.7	(*3, 4, 5*)

* All values on dry basis.

TABLE II

The Protein Composition of Various Preparations

Preparations	Phosvitin	α-Lipovitellin*	β-Lipovitellenin*	Other components*
	%			
0.09 *M* Ppt*	40–44	21–24	0	34–37
Phosvitin	100	0	0	0
Lipovitellin	16–18	36–40	26–29	16–19
Lipovitellenin	0	10–13	85–90	trace

* See Text.

Preliminary Study of Isolation of α-Lipovitellin from the Lipovitellin Preparation of Alderton and Fevold

The α-lipovitellin could be isolated from the lipovitellin prepara-

---

2) As shown in the latter section, the lipid contents of β-lipovitellin is about 40 per cent.

tion of Alderton and Fevold by the following method. The fraction, which was separated from the diluted yolk (1 part yolk to 2 parts water) by centrifugation, was dissolved at once into a small amount of 1.2 M magnesium sulfate solution, diluted by the careful addition of water until 0.09 M, and allowed to stand overnight. After removing the precipitate by centrifugation, solid sodium chloride was added to the supernatant solution to 5 to 10 per cent. The solution was shaken with ether to remove the uncombined lipid completely[3] and then dialyzed against streaming water. The obtained precipitate was suspended into water, dissolved by adding 0.1 N sodium hydroxide, and precipitated by 0.1 N sulfuric acid. The precipitate, in this case, could be dissolved at pH 9.3 to 9.6 and precipitated at pH 7.5 to 7.8[4]. After the reprecipitation was repeated three times, the α-lipovitellin showed an electrophoretic pattern as Fig. 5. In addition to the major component having a mobility of -5.3 units, a small amount (7 to 8 per cent) of minor component having a mobility of -4.4 units could be observed. All components which have larger mobility than -5.3 units were removed perfectly by the magnesium sulfate dilution method.

A similar product of α-lipovitellin could be obtained also by another method. When the supernatant from the 0.09 M magnesium sulfate precipitate was extracted at once with ether without adding sodium chloride, a white precipitate layer was formed between the ether and aqueous layers[5]. This precipitate was collected by centrifugation, washed centrifugally by water, and then purified by the acid-alkali reprecipitation method as stated above[6]. This product contained an almost equal amount of minor component and showed a similar electrophoretic pattern to the one in Fig. 5.

The solubility of α-lipovitellin became lower gradually by repeating the acid-alkali reprecipitation many times ; namely, the pH which began to dissolve shifted toward the alkaline side gradually and some turbidity remained in the solution. Therefore, it was discontinued to purify the α-lipovitellin further by repeating acid-alkali reprecipitation.

---

[3] In this case, the color does not shift easily to the ether layer, but mostly shifts after standing overnight. The ether extraction was repeated further several times.

[4] Lowering the pH further, the precipitate dissolves again at the pH of acidic side (about 4.5).

[5] If the extraction is carried out below 15°, a larger quantity of precipitate is formed and the coprecipitating β-lipovitellin increases.

[6] Unless the salt is removed thoroughly by washing, the precipitate could not be dissolved by adding alkali.

The obtained α-lipovitellin contained 0.7 to 0.8 per cent phosphorus and 6.5 to 7.0 per cent lipid. Assuming the minor component to be β-lipovitellin, it is inferable that α-lipovitellin contains 3 to 4 per cent lipid. The study on the isolation of α-lipovitellin by another method is in progress at this laboratory.

Lipovitellenin Preparation of Fevold and Lausten

The contents of lipid and phosphorus of the lipovitellenin prepared by the method of Fevold and Lausten (*4*) are given in Table I. Both those values and those reported in the original favorably agreed. From the decrease of phosphorus content after alcohol extraction, it was estimated that about 83 per cent of phosphorus in this preparation was attributed to the phospholipid.

An electrophoretic pattern of the lipovitellenin is shown in Fig. 6. The protein composition is given in Table II. The phosvitin component could not be found in this preparation, but, when the lipovitellin fraction was removed by centrifugation at 3,000 r.p.m., it was recognized. This preparation was far richer in main component than the lipovitellin. Almost of the minor component which consisted 10 to 13 per cent had a mobility equal to the α-lipovitellin. Therefore, it could be considered that the pure β-lipovitellin contains about 40 per cent lipid.

The similar preparation with the lipovitellenin could be obtained also by the following two methods. When the supernatant of the lipovitellin was dialyzed at once against water, the yellow precipitate was obtained. This was dissolved in 10 per cent sodium chloride solution, extracted the uncombined lipid with ether, and dialyzed again. In another method, solid sodium chloride was added to the supernatant of the lipovitellin to 10 per cent, extracted with ether, and dialyzed against water. Both preparations were similar with the lipovitellenin in the electrophoretic composition and the contents of lipid and phosphorus.

The lipovitellenin could be purified to a certain extent by repeating the acid-alkali reprecipitation, but its solubility reduced gradually as well as in the case of the α-lipovitellin. In the lipovitellenin, it could be dissolved and precipitated at the pH of more acidic side than the α-lipovitellin ; namely, it was dissolved at pH 8.0 to 8.5 and precipitated at pH 6.5 to 7.0. It was also possible to dissolve it at the pH of acidic side (4.5 to 5.0).

SUMMARY

1. Concerning the nature of phosvitin and its isolation process, some investigations were carried out by using an electrophoretic method. The mobility of phosvitin was −11.3 units at pH 9.8 and μ 0.15. The phosvitin represented an average of 8.5 per cent of the yolk protein. The lipid and phosphorus contents were in good agreement with the results of Mecham and Olcott. The solubility property of the isolated phosvitin was stated.

2. The homogeneity of the lipovitellin prepared by the method of Alderton and Fevold was studied electrophoretically. This preparation was not homogeneous and its protein composition was given. Two provisional names, " α-lipovitellin " and " β-lipovitellin ", were used for two main electrophoretic components of the yolk protein, which had the mobilities of −5.3 and −4.4 units, respectively. About 72 per cent of the total phosphorus and about 83 per cent of the protein phosphorus of the lipovitellin were attributed to the 16 to 18 per cent phosvitin which was contained in it. And similarly, about 81 per cent of the lipid of the lipovitellin was accounted for β-lipovitellin which was involved 26 to 29 per cent in it.

3. The preliminary study on the isolation of α-lipovitellin from the lipovitellin was reported. The isolated α-lipovitellin preparation showed the purity over 90 per cent and it contained 0.7 to 0.8 per cent phosphorus and 6.5 to 7.0 per cent lipid. The solubility property of this preparation was stated.

4. The lipovitellenin prepared by the method of Fevold and Lausten was studied by the electrophoretic method. As compared with the lipovitellin, this preparation was considerably pure; namely, over 85 per cent of it consisted of the β-lipovitellin and the phosvitin was not contained. About 83 per cent of phosphorus of the lipovitellenin was due to the phospholipid which was alcohol-extractable.

The author wishes to express his thanks to Mr. K. Terao and Miss. I. Watanabe for their technical assistances in carrying out the experiments.

REFERENCES

(*1*) Osborne, T. B. and Campbell, G. F., *J. Am. Chem. Soc.*, **22**, 413 (1900)
(*2*) Chargaff, E., *J. Biol. Chem.*, **142**, 491 (1942)
(*3*) Alderton, G. and Fevold, H. L., *Arch. Biochem.*, **8**, 415 (1945)
(*4*) Fevold, H. L. and Lausten, A., *Arch. Biochem.*, **11**, 1 (1946)

(*5*) Mecham, D. K. and Olcott, H. S., *J. Am. Chem. Soc.*, **71**, 3670 (1949)
(*6*) Mecham, D. K. and Olcott, H. S., *Fed. Proc.*, **7**, 173 (1948)
(*7*) Sugano, H., *Seibutu-Buturi-Kagaku*, **1**, 118 (1952) (in Japanese) ; *J. Fac. Sci. Niigata Univ., Ser. 1*, **1**, 41 (1955) (in English)
(*8*) Allen, R. J. L., *Biochem. J.*, **34**, 858 (1940)

The Journal of Biochemistry, Vol. 44, No. 4, 1957

ON THE OXIDATION OF NON-HYDROXYAMINO ACIDS BY PERIODIC ACID

By KIKUO ARAKAWA

(From the Department of Medical Chemistry, Faculty of Medicine, Kyushu University, Fukuoka)

(Received for publication, December 19, 1956)

It has been known since the report of B. H. Nicolet and L. A. Shinn (*1*) that periodic acid is capable of oxidizing β-hydroxy-α-amino acids to convert them into ammonia, corresponding aldehyde, and glyoxylic acid. This reaction of periodic acid makes it possible to determine serine and threonine quantitatively by treating them with the acid in a protein hydrolysate and measuring the formaldehyde and acetaldehyde produced (*2*, *3*, *4*).

It was also applied by D. D. Van Slyke to the determination of hydroxylysine. He asserted that under the condition he adopted (25 minutes at room temperature) non-hydroxyamino acids, when acted on by an alkaline periodate, gave no appreciable amount of ammonia.

It seemed to us at first that the reaction is somewhat promising to cleave a peptide- or protein chain such as protamine at every hydroxyamino acid content. The present communication deals with the results of some experiments on the oxidation of amino acids without hydroxy group by means of periodic acid, which have been carried out under a more drastic condition than that of Van Slyke, with especial reference to the oxidation velocities and fragments of amino acids.

EXPERIMENTAL

1. Oxidation Velocities

Method—Each amino acid was oxidized in a neutral medium, so that it might be readily compared with the experiment of peptide*. As a standard procedure, 5–10 mg. of amino acid dissolved in 1 ml. of water, 1 ml. of 0.5 *M* periodic acid solution, and 20 ml. of *M*/25 phosphate buffer of pH 8.03 were put together into an ampule. The pH of the mixture ranged generally from 6.9 to 7.0 at this stage. The sealed ampule, kept at room temperature or 60° for some periods, was cooled rapidly in ice water and opened. The content was, then, added with an excess of barium acetate solution and kept at 0° for one hour. Barium periodate precipitated was filtered off and the residue was washed three times with a few ml. portion of cold water. The filtrate and washings were combined, made alkaline in Parnas' apparatus, and distilled for the quantitative determination of ammonia or methylamine.

---

* Unpublished experiment.

Results—Both serine and threonine were readily attacked at room temperature, the whole substance being oxidized within 30 minutes.

Ammonia, on the contrary, was not liberated from non-hydroxyamino acids in measurable amount under the same condition. The minute quantity of ammonia liberated in 24 hours is shown in Table I. The oxidation became more markedly when the temperature was raised to 60°. Experiments with different amino acids under this condition disclosed that the time required for oxidation was in inverse proportion to the length of straight carbon chains in the case of monoamino- and diamino-monocarboxylic acids (Fig. 1, 2) and the reverse was the case in that of monoamino-dicarboxylic acids (Fig. 3). β-Amino acids are more highly resistant to periodic acid than α-amino acids as shown in Fig. 1. In additional experiments with methionine under the same condition, no difference could be observed between DL- and L-forms in their oxidation velocities (Fig. 3).

Table I

Ammonia Liberated from Non-hydroxyamino Acids by the Action of Neutral Periodate at Room Temperature for 24 Hours

Amino acids	$\frac{\text{Librtd. N}}{\text{Total N}}$ (%)	DL-Val	8.0	L-Asp	21.8
Gly	3.7	DL-*n*-Leu	12.0	L-Glu	11.5
α-Ala	5.1	L-ILeu	14.4	DL-Phe	16.8
β-Ala	1.6	L-Leu	19.3	L-Dopa	20.0
α-Amibut	8.5	L-Orn	6.8	L-His	17.7
β-Amibut	4.4	L-Lys	12.6	L-Try	15.3
DL-*n*-Val	6.5	L-Arg	5.5	DL-Met	11.9

5–10 mg. amino acid dissolved in 1 ml. of water and 20 ml. of *M*/25 phosphate buffer (pH 8.03) was oxidized in ampule with 1 ml. of 0.5 *M* HIO_4 at room temperature. After 24 hours, excess of HIO_4 was removed as barium salt. The filtrate and washings were combined and ammonia was determined as described in text.

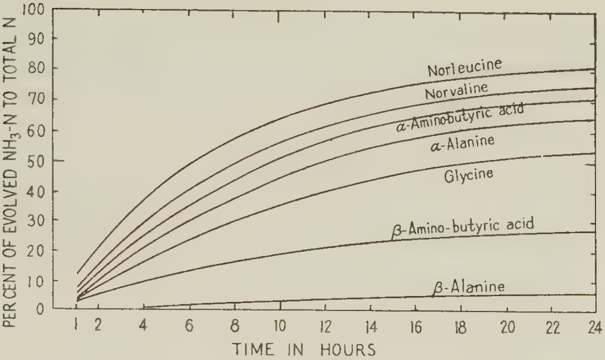


FIG. 1. Ammonia liberation by the action of periodic acid at 60° from aliphatic amino acids with straight carbon chains and their position isomers.

The same reaction mixtures as described in Table I were oxidized in water bath of 60°. Ammonia liberated from them were measured also in the same way every 2 hours until 24 hours.

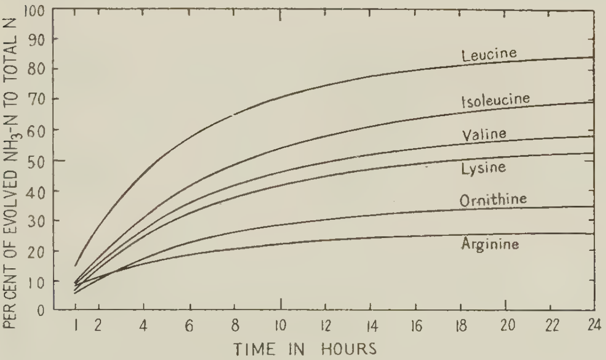


FIG. 2. Ammonia liberation by the action of periodic acid at 60° from diamino-monocarboxylic acids and chain isomers of aliphatic amino acids (for the explanation see Fig. 1.).

N-Methylamino acids, from which methylamine, not ammonia, is liberated as will be described later, are somewhat below the corresponding ordinary amino acids in oxidation velocities (Table II).

2. *Oxydation Products*

Amino Group—The oxydation products of amino acid and *N*-methylamino acid, when distilled into Nessler's reagent with Parnas' apparatus, presented a brown color and a golden color, and when into hydrochloric acid, yielded ammonium hydrochloride and methylamine hydrochloride, respectively. The latter was identified by its mixed melting point with an authentic specimen.

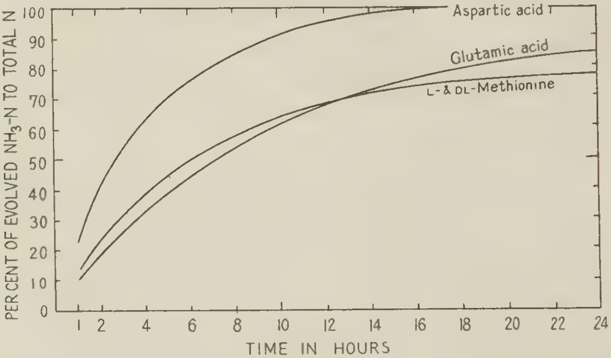


FIG. 3. Ammonia liberation by the action of periodic acid at 60° from monoamino-dicarboxylic acids and geometrical isomers of methionine (for the explanation see Fig. 1.).

TABLE II

Methylamine Liberated from N-Methylamino Acids by the Action of Neutral Periodate at 60° for 24 Hours

N-Methyl-amino acids	$\frac{\text{Librtd. N}}{\text{Total N}}$ (%)	Amino acids	$\frac{\text{Librtd. N}}{\text{Total N}}$ %
N-Methyl-DL-*n*-val	68.6	DL-*n*-Val	75.8
N-Methyl-DL-*n*-leu	78.7	DL-*n*-Leu	80.9
N-Methyl-L-leu	83.6	L-Leu	85.2
N-Methyl-L-lys	45.6	L-Lys	53.8

Methylamine was also measured by the same procedure as described in Table I.

Carboxyl Group—The fact that non-hydroxyamino acid is decarboxylizable by the action of periodate was confirmed by measuring by the Van Slyke method (*6*) the amount of carbon dioxide split off. The internal pressure of the apparatus was low, so that there was a gradual decline in the amount of the reaction mixture, and the evolving velocities of carbon dioxide was not determinable over so long a period as in the case of ammonia. But the rates of producing carbon dioxide were nearly equal to that of ammonia evolution until 4 hours, as will be seen when Table III and Figs. 1–4 are compared.

Residual Part—After the removal of excess periodate, the remainder was next distilled, without being made alkaline, for identification of the major skeleton of the amino acids. The distillates, which were positive to tests with Schiff's and Fehling's reagent, gave dimedon derivatives and 2–4 dinitro-phenylhydrazones. Their mixed melting point with authentic specimens were determined or their elementary analyses were made to identify the original aldehyde. Their amount were determined either colorimetrically using chromotropic acid according to B. Alexander (*7*) or gravimetrically using dimedon and 2–4 dinitro-phenylhydrazine. For the gravimetrical determinations were required 250 mg. or more of amino acid, 5 ml. of 0.5 M HIO_4, and 100 ml. of phosphate buffer of pH 8.03. The results of the analyses were as follows:

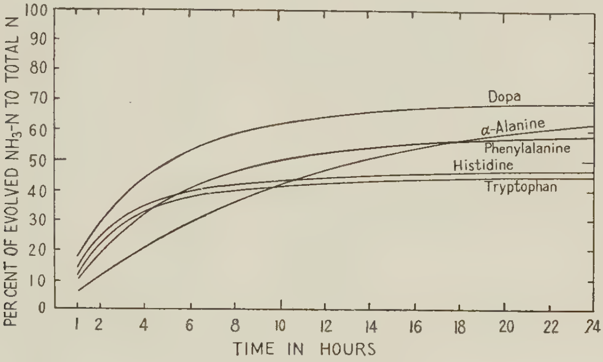


FIG. 4. Ammonia liberation by the action of periodic acid at 60° from amino acids related to alanine (for the explanation see Fig. 1.).

The amount of HCHO obtained by the oxidation of 10 mg. of glycine at 60° for 24 hours was 1.03 mg., that is, 27.5 per cent of the calculated amount—a percentage lower than that in which NH_3 was obtained under the same conditions. About the same was the case with the oxidation of serine at 60° for 24 hours, the amount of HCHO obtained in this case not exceeding 39.7 per cent of the calculated, while it reached 100 per cent in the oxidation of serine at room temperature for one hour.

Aldehydes obtained from the other amino acids are shown in Table IV.

DISCUSSION

Non-hydroxyamino acids are oxidized only in a prolonged period of time and at high temperature. Under such conditions, however, the yield of their respective corresponding aldehyde, even that of HCHO from serine, is markedly low in value as compared with that of NH_3 evolved. But, NH_3 and CO_2 are liberated at an equal rate, suggesting that there may be a simultaneous quantitative production of aldehyde. It is therefore conceivable that the aldehyde once produced may change by degrees into another substance under the said conditions.

TABLE III

Carbon Dioxide Liberated from Non-hydroxyamino Acids by the Action of Periodic Acid at 60° for 1–4 Hours

Amino acids	$\frac{\text{C librtd. as CO}_2}{\text{Total carboxyl C}}$ (%)		
	1 h.	*2 hs.*	*4 hs.*
Gly	6.9	9.5	17.3
α-Ala	7.1	11.0	21.7
β-Ala	0.7	1.2	3.2
α-Amibut	9.0	14.8	29.3
β-Amibut	1.9	3.0	5.3
n-Val	10.5	18.3	37.5
Val	10.2	19.4	29.7
n-Leu	15.9	26.2	35.5
ILeu	16.1	20.5	34.9
Leu	29.2	34.0	57.7
Orn	9.7	13.9	20.7
Lys	6.9	14.4	26.8
Arg	6.3	14.1	25.4
Asp	19.5	37.3	63.4
Glu	10.7	16.4	34.5
N-Methyl-*n*-val	8.8	15.7	33.4
N-Methyl-*n*-leu	13.5	24.1	34.8

Two little flasks and a U-shaped glass tube were connected with rubber tubes. After the reaction mixture (10 mg. amino acid dissolved in a mixture of 1 ml. of water, 1 ml. of 0.5 *M* HIO_4, and 20 ml. of *M*/25 phosphate buffer of pH 8.03) and 5 ml. of 0.1 *N* $Ba(OH)_2$ were poured into each flasks, then the air in the apparatus was removed immediately. One flask containing reaction mixture was dipped in water bath of 60° and the receiving flask containing $Ba(OH)_2$ was placed in cold water bath shaking frequently. After 1, 2, and 4 hours, the residual $Ba(OH)_2$ were titrated with 0.1 *N* HCl.

TABLE IV

Aldehydes obtained from Non-hydroxyamino Acids by the Action of Neutral Periodate at 60° for 24 Hours

Amino acid	Expected aldehyde	Kinds of derivative	M.p. of the derivative (C)	M.p. of standard preparation (C)	M.p. of the mixed (C)	Yields of aldehyde to the calculated (%)	Confirmed aldehyde
α-Ala	acetaldehyde	dimedon	136–138	138–139	136–138	16.9	acetaldehyde
α-Amibut	propionaldehyde	dimedon	153	155	153–155	15.4	propionaldehyde
n-Val	*n*-butylaldehyde	dimedon	139–140	142	139–141	16.2	*n*-butylaldehyde
N-Methyl-*n*-val	*n*-butylaldehyde	DNPH 1)	121	122	120–121	18.9	*n*-butylaldehyde
Val	*i*-butylaldehyde	dimedon	151–153	154	152–153	12.5	*i*-butylaldehyde
n-Leu	*n*-valeraldehyde	DNPH	98	98	98	23.5	*n*-valeraldehyde
N-Methyl-*n*-leu	*n*-valeraldehyde	DNPH	97–98	98	97	21.8	*n*-valeraldehyde
Leu	*i*-valeraldehyde	dimedon	150–151	155	149–153		*i*-valeraldehyde
,,	,,	DNPH	121	123	122–123	20.6	,,
ILeu	methylethylacetaldehyde	DNPH	110–111		2)	15.4	methylethylacetaldehyde
Asp	semialdehyde of malonic acid	dimedon	134–136		3)	16.3	acetaldehyde
Glu	semialdehyde of succinic acid	dimedon	154		4)	9.7	propionaldehyde
Phe	phenylacetaldehyde	DNPH	233–234	110	5)	11.6	benzaldehyde

1) " 2–4 Dinitrophenylhydrazone " is abbreviated as DNPH.

2) DNPH of methylethyl-acetaldehyde was confirmed by the elementary analysis.

		C%	H%	N%
$C_{11}H_{14}O_4N_4$ (266.25)	calcd.	49.62	5.30	21.04
	found	49.57	5.42	20.98

3) The derivative of the aldehyde obtained from aspartic acid showed m.p. of acetaldehyde derivative, and its mixed m.p. with standard acetaldehyde derivative showed no depression.

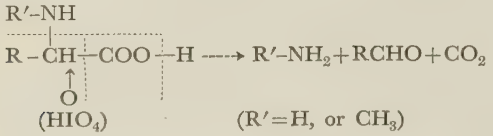
4) The aldehyde from glutamic acid was confirmed in similar way, and also by N analysis.

$$N\begin{cases}\text{calcd. as dimedon derivative of } HOOCCH_2CH_2CHO: & 19.85\% \\ \text{,, as dimedon derivative of } CH_3CH_2CHO: & 23.52\% \\ \text{found}: 23.72\% & \end{cases}$$

5) The derivative of the aldehyde obtained from phenylalanine was confirmed as that of benzaldehyde by means of similar mixed melting point test and N analysis.

$$N\begin{cases}\text{calcd. as DNPH of } C_6H_5CH_2CHO: & 18.66\% \\ \text{,, as DNPH of } C_6H_5CHO: & 19.58\% \\ \text{found}: 19.45\% & \end{cases}$$

It is evident from the above-mentioned results of the present experiments that, of the non-hydroxyamino acids, monoamino-monocarboxylic acids with straight carbon chains and that with branched chains are oxidized primarily in accordance with a scheme like the following:



$$\mathrm{R-\overset{\displaystyle R'-NH}{\underset{\underset{\displaystyle O}{\uparrow}}{CH}}-COO-H} \dashrightarrow \mathrm{R'-NH_2+RCHO+CO_2}$$

(HIO_4) $(R'=H, \text{ or } CH_3)$

It is to be expected that aspartic acid yields semialdehyde of malonic acid and that glutamic acid yields semialdehyde of succinic acid. In reality, however, it has been demonstrated experimentally (Table IV) that they yield acetaldehyde and propionaldehyde, respectively. The fact makes it most probable that, whatever the reaction mechanism may be, monoaminodicarboxylic acids may be decomposed by the action of periodic acid at 60° as follows:

$$\mathrm{HOOC{\cdot}R{\cdot}CH(NH_2){\cdot}COOH} \dashrightarrow \mathrm{CO_2+HR{\cdot}CHO+NH_3+CO_2}$$

On the other hand, there may be some other patterns of amino acid oxidation by the action of periodic acid because phenylalanine yields benzaldehyde instead of phenylacetaldehyde as Table IV shows unexpectedly, and no aldehyde was obtained from diamino acids which oxidized and treated in the same procedure as described above.

SUMMARY

1. Many non-hydroxyamino acids were oxidized by neutral periodate at 60° for 24 hours to be converted into NH_3, CO_2, and their respective corresponding aldehyde, but not so at any appreciable rate at room temperature.

2. The oxidation velocities of a variety of non-hydroxyamino acids were determined until 24th hour and compared with one another.

3. The oxidation patterns of some amino acids were discussed.

The author wishes to express his sincere gratitude to Prof. Dr. R. Hirohata, director of the Department, and Prof. Dr. M. Tomoda, director of the Department of 2nd Surgical Clinic, for their careful guidance given throughout the present research.

REFERENCES

(*1*) Nicolet, B. H., and Shinn, L. A., *J. Am. Chem. Soc.*, **61**, 1615 (1935)
(*2*) Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **35**, 294 (1941)
(*3*) Rees, M. W., *Biochem. J.*, **40**, 632 (1946)
(*4*) Boyd, M. J., and Logan, M. A., *J. Biol. Chem.*, **146**, 279 (1942)
(*5*) Van Slyke, D. D., Hiller, A., and MacFadyen, D. A., *J. Biol. Chem.*, **141**, 681 (1941)
(*6*) Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 671 (1941)
(*7*) Alexander, B., Landwehr, G., and Seligman, A. M., *J. Biol. Chem.*, **160**, 51 (1945)

The Journal of Biochemistry, Vol. 44, No. 4, 1957

UNUSUAL STRUCTURES IN CORN STARCH GRANULES*

By ZIRO NIKUNI and ROY L. WHISTLER

(From the Institute of Scientific and Industrial Research, Osaka University, Osaka and the Department of Biochemistry, Purdue University, Lafayette, Indiana, U.S.A.)

(Received for publication, January 9, 1957)

The surface and internal structure of starch granules from corn and other sources have been examined previously with the aid of the electron microscope (*1–4*). Sections 0.05 to 0.2 microns thick are seen to possess rather smooth internal structures with a fine granulation and in addition may show concentric lamellar rings surrounded by a somewhat irregular periphery (*1*, *2*).

Infrequently holes and saw-toothed fissures are observed in thin sections (*4*). Details of such structure can be seen in serial sections of starch granules (*5*). Some irregularities in the form of holes or cracks occur in about one-fourth of all corn starch granules. The present paper shows that certain of these granules irregularities can be simulated by action of salivary amylase. Possibly attack of plant enzymes on starch granules *in vivo* may account for some of the pits or borings which are observed in isolated starches.

EXPERIMENTAL

(*1*) *Preparation of the Specimens*—

a. Commercial Corn Starch: Commercial Buffalo corn starch granules were embedded for sectioning in a mixture of 20 per cent methyl methacrylate in *n*-butyl methacrylate either by vacuum embedding or by passing through a water-alcohol-methacrylate sequence (*1*, *4*, *5*).

b. Digested Corn Starch: One gram (12 per cent moisture basis) of commercial Buffalo corn starch was digested by 10 ml. of 1:20 fresh human saliva for 24 hours at 30°. After washing and drying, 0.6 g. of the partially digested starch granules were embedded in methacrylate as above.

---

* This work was done in the Department of Biochemistry, Purdue University, Lafayette, Indiana, U.S.A. Journal Paper No. 1052 of the Purdue Agricultural Experimental Station.

c. Starch Granules in Corn Endosperm: Common yellow field corn, single cross (Wf-9X38-11) was grown in field plots of Purdue University and was pollinated and the kernels killed, fixed (*6*), dehydrated and embedded as previously described (*4*, *5*).

(*2*) *Sectioning*—

Embedded specimens were sliced with a wedge-modified Spencer Model 820 rotary microtome using glass-knives. The thickness of the sections was adjusted from 0.05 to 0.25 microns as needed.

(*3*) *Leaching and Shadowing*—

Usually, a ribbon of sections was transferred onto a supporting grid from the surface of a solution (dioxane : water, 1:1 v/v) contained in a pool on the glass-knife. The methacrylate embedding material was extracted from the dried specimen by submerging the specimen in toluene for 10 minutes. Supporting grids were previously covered by a silicon dioxide membrane (*4*). Specimens on grids were chromium shadowed at an 18° angle.

(*4*) *Digestion of Thin Sections of Commercial Corn Starch Granules*—

A section of ordinary commercial corn starch, 0.25 microns in thickness, was transferred on to silicon dioxide covered grid as described above except that the specimen was not leached by toluene.

The grid with the specimen was soaked in salivary amylase solution for a definite period and was then washed carefully by dipping into 25 per cent and 50 per cent ethanol, successively, before air drying. The period of digestion was varied from 30 seconds to 4 hours. Usually one minute's digestion was sufficient to produce substantial effects. Following salivary digestion and drying sections were leached with toluene to remove the methacrylate resin and were shadowed at an 18° angle by chromium.

Salivary amylase solution was prepared as follows : about 5 ml. of fresh human saliva was diluted with the same volume of water, centrifuged twice and filtered. The almost clear filtrate thus obtained was used without addition of buffer.

The enzyme solution was very active. When corn starch granules were treated with this solution and observed under the light microscope they were seen to be rapidly swelled and digested. After approximately 3 hours almost all the granules were digested and disappeared.

RESULTS AND DISCUSSION

A normal undigested section of a commercial corn starch granule is shown in Fig. 1. The internal structure of the corn starch granules seems to contain fine granular particles. The concentric lamellar structure is obvious in this section, but the lamellar rings are found in only one-fifth to one-seventh of the sections observed.

Sections with central cavities and irregular saw-toothed fissures are found occasionally in ordinary commercial corn starch. Examples of such uncommon cavities are shown in Figs. 2 and 3. It is quite possible that these fissures are caused by partial digestion of the starch

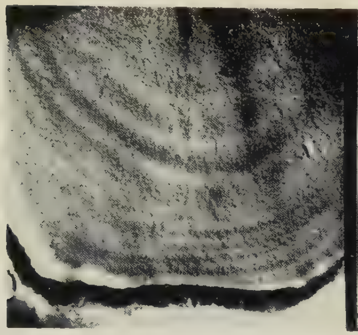


FIG. 1. Normal section of corn starch granule with rings. ×8,050.

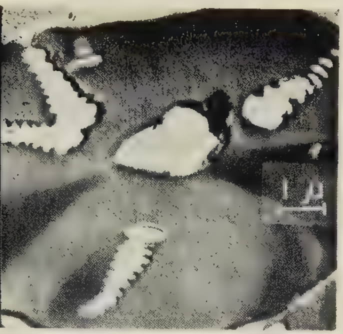


FIG. 2. Section of corn starch granule with hole and fissures. ×5,830.

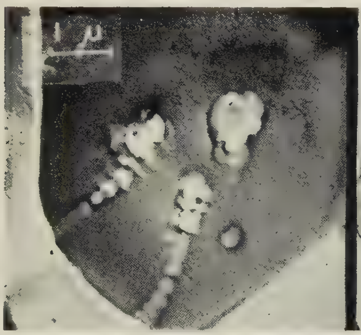


FIG. 3. Section of corn starch granule with hole and fissures. ×8,050.

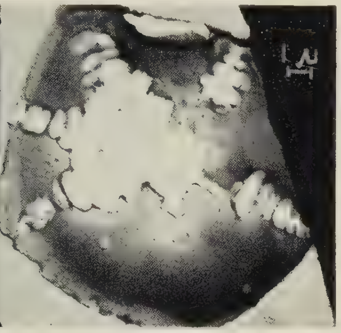


FIG. 4. Section of digested corn starch granule. ×3,800.

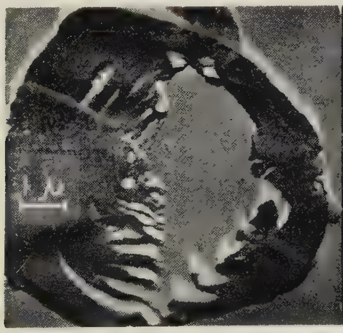


FIG. 5. Section of digested corn starch granule. ×5,830.

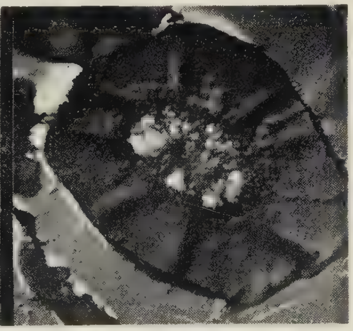


FIG. 6. Section of corn starch granule in endosperm, 66 days after pollination. ×3,800.

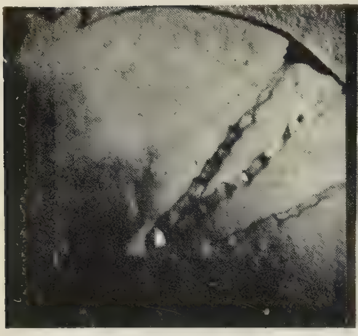


FIG. 7. Digested section of corn starch granule with radial canals. ×8,050.

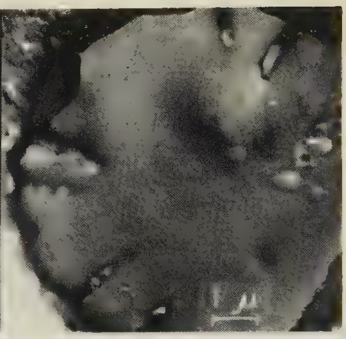


FIG. 8. Digested section with radial canals. ×5,830.

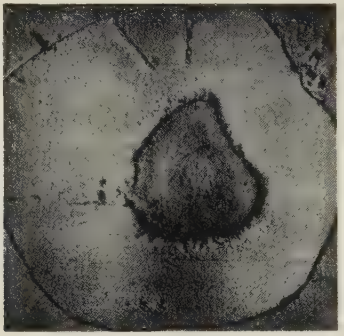


FIG. 9. Digested section with loose part and canals. ×3,800.

granule before removal from the kernel. Conceivably some amylolytic hydrolysis could occur *in vivo* before harvest, or even after harvest through the agency of natural endogenous enzymes or contaminating mold enzyme. Whatever the source of the enzymes, sattack likely begins at a weak or susceptible spot on the granule surface and proceeds inward along an accesible pathway of low density or of less organized starch molecules. The ragged tooth-like shape of the radial pits may be due to different densities present in visible and invisible lamellar rings. Since many starch granules seem to have a low density or weak central core this part of the granules is readily attacked and hydrolyzed by amylolytic enzymes as shown in Figs. 4, 5 and 6.

Approximately half of the sections digested with salivary amylase developed patterns similar to those seen in rare slices taken from commercial and laboratory isolated corn starch (Figs. 4 and 5).

Figs. 7, 8 and 9 show the shapes of radial canals and central cavities which were brought into relief when salivary enzyme acted on thin specimens partially fixed with methacrylate resin. While the resin inhibits the enzyme attack, sections freed of resin are swelled or dissolved by water and almost instantaneously disappear on addition of enzyme.

SUMMARY

Electron microscopic observation of ultra-thin sections of certain corn starch granules shows fissures and borings which are similar to those produced in methacrylate impregnated sections treated with salivary amylase.

REFERENCES

(*1*) Whistler, R. L., Byrd, J. D., and Thornburg, W. L., *Biochim. et Biophys. Acta*, **18**, 146 (1955)
(*2*) Whistler, R. L., Turner, E. S., *J. Polymer Sci.*, **18**, 153 (1955)
(*3*) Kore-eda, A., Nikuni, Z., Memoirs of the Inst. of Sci. and Ind. Research, Osaka University, XII, 141 (1955)
(*4*) Whistler, R. L., Thornburg, W. L., *J. Ag. Food Chem.*, in press.
(*5*) Whistler, R. L., Nikuni, Z., unpublished.
(*6*) Sass, J. E., *Stain Technol.*, **20**, 93 (1945)

The Journal of Biochemistry, Vol. 44, No. 4, 1957

BIOCHEMICAL STUDIES ON SULFATE-REDUCING BACTERIA

VI. SEPARATION OF HYDROGENASE AND THIOSULFATE REDUCTASE AND PARTIAL PURIFICATION OF CYTOCHROME AND GREEN PIGMENT*

By MAKOTO ISHIMOTO AND JIRO KOYAMA**

(*Department of Chemistry, Faculty of Science, University of Tokyo, Tokyo*)

(Received for publication, January 16, 1957)

In the previous papers, the authors reported extraction of hydrogenase, dehydrogenases and thiosulfate reductase, as well as of a cytochrome with low potential and a green pigment from cells of the sulfate-reducing bacteria by grinding with alumina or silica (*1*, *2*). They found that thiosulfate reduction by the extracts, supplemented with methyl viologen, took place in two steps, methyl viologen was reduced with hydrogen by hydrogenase, and then the reduced methyl viologen was oxidized with thiosulfate by thiosulfate reductase. In the present paper, it is reported that this mechanism of the reaction was confirmed by separation of the enzymes and by reconstruction of the thiosulfate reducing system. Partial purification of the cytochrome and the green pigment was also intended.

MATERIALS AND METHODS

Preparation of Cell-Free Extracts—The strain and the procedure to prepare the cell-free extracts of sulfate-reducing bacteria were same as reported in the previous papers (*1*, *3*).

Assay of Hydrogenase Activity—Manometric measurement of hydrogen up take was carried out with methylene blue as a hydrogen acceptor. The reaction mixtures contained 0.2 ml. of 3 per cent ovalbumin solution, 1.5 ml. of 0.067 *M* phosphate buffer pH 7.0, and 0.5 ml. of samples for assay in the main compartments, 0.3 ml. of 0.01 *M* methylene blue in the side arms and 0.2 ml. of 20 per cent potassium hydroxide in the center wells. Ovalbumin solution was used as a diluent, because the hydrogenase solution from sulfate-reducing bacteria was unstable for dilution and lost its activity immediately by heavy dilution with buffer solution. When diluted with ovalbumin solution, the rates of the hydrogen consumption were nearly proportional to the amounts of hydrogenase. The rates of the hydrogen uptake in steady state were expressed in μ moles per hour.

---

* This paper was read at the 7th Symposia on Enzyme Chemistry in July, 1954.

** Present address ; Institute for Infectious Diseases Department of Biological Physics and Chemistry, University of Tokyo, Tokyo.

Assay of Thiosulfate Reductase—The measurement depended on the fact that blue color of reduced methyl viologen disappeared by oxidation with thiosulfate in the presence of the enzyme. The reaction was carried out in the Thunberg tubes under anaerobic condition and the decolorization time was measured. The reaction mixtures contained 0.5 ml. of 0.067 *M* phosphate buffer, pH 7.0, within 1.0 ml. of samples for assay and 5.0 ml. of reduced methyl viologen 0.002 *M* in 0.013 *M* phosphate buffer, pH 7.0, in the tubes and 0.5 ml. of 0.1 *M* sodium thiosulfate in the hollow stoppers. As a unit of thiosulfate reductase, μ moles of reduced methyl viologen, oxidized with thiosulfate per hour, were calculated from the decolorization time, for these values were nearly proportional to the amounts of the extracts.

Determination of the Spectra and Assays of the Cytochrome and the Green Pigment—Beckman spectrophotometer, Model DU, was employed with cells of 1 cm. optical path for the determination of the spectra.

As the cytochrome has an absorption maximum at 553 mμ and a minimum near 540 mμ in the reduced form (*1*), the difference of the optical density at 553 mμ and at 540 mμ was used as a unit of the cytochrome concentration. The difference of the optical density at 553 mμ and at 540 mμ is approximately seven tenths of the optical density at 553 mμ in the reduced form.

For green pigment, the difference of the optical density at 630 mμ and at 620 mμ was tentatively defined as a unit.

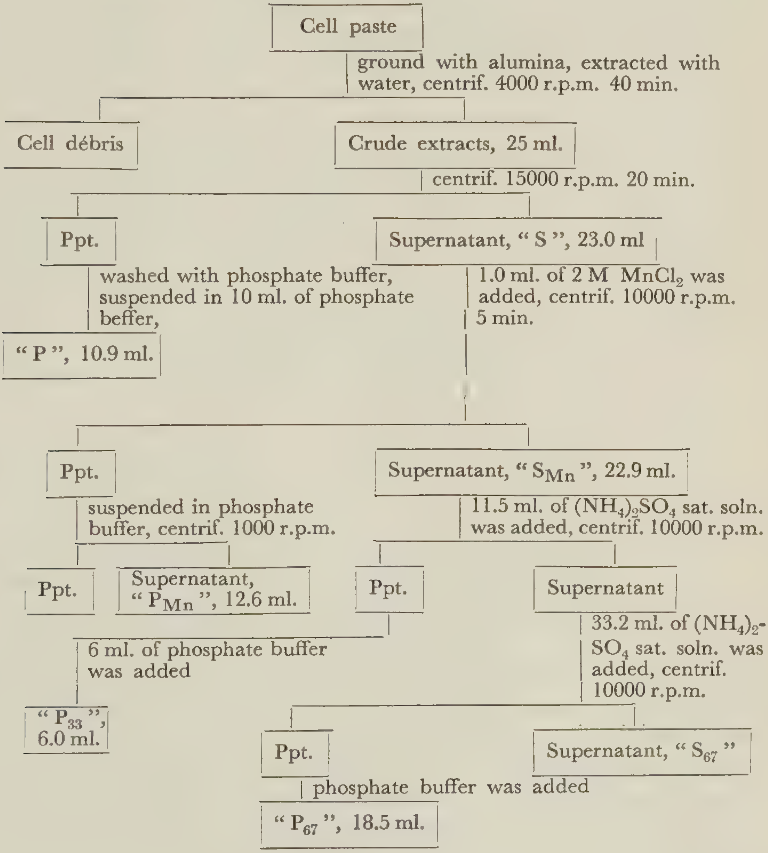
EXPERIMENTALS AND RESULTS

Fractionation with Ammonium Sulfate—The cells of the sulfate-reducing bacteria were ground with alumina, extracted with water or saline and centrifuged at 4000 r.p.m. for 40 minutes to remove cell débris and particles of alumina. The extracts were further centrifuged at 16000 r.p.m. for 20 minutes. The gray precipitate was washed once with 0.067 *M* phosphate buffer, pH 7.0, and suspended in the same buffer (P). The supernatant (S) was dark brown. For the purpose of elimination of nucleic acid, the presence of which would interfere the further treatment, a twentieth volume of 2 *M* manganese chloride was added drop by drop, maintaining the solution at pH 7.0 with alkali. The gray precipitate formed was removed by centrifugation and extracted with phosphate buffer (P_{Mn}). The supernatant (S_{Mn}) was then treated with ammonium sulfate for fractionation. The fractions precipitated at 33 per cent (P_{33}) and between 33 per cent and 67 per cent (P_{67}) saturation with ammonium sulfate were taken respectively. An example of the procedure is shown in Diagram 1.

The results of the determination of hydrogenase and thiosulfate reductase activities contained in each fraction are listed in Table I.

DIAGRAM 1

Fractionation of Extracts of Sulfate-reducing Bacteria with Ammonium Sulfate


Cell paste
ground with alumina, extracted with water, centrif. 4000 r.p.m. 40 min.
Cell débris
Crude extracts, 25 ml.
centrif. 15000 r.p.m. 20 min.
Ppt.
Supernatant, " S ", 23.0 ml
washed with phosphate buffer, suspended in 10 ml. of phosphate beffer,
" P ", 10.9 ml.
1.0 ml. of 2 M $MnCl_2$ was added, centrif. 10000 r.p.m. 5 min.
Ppt.
Supernatant, " S_{Mn} ", 22.9 ml.
suspended in phosphate buffer, centrif. 1000 r.p.m.
11.5 ml. of $(NH_4)_2SO_4$ sat. soln. was added, centrif. 10000 r.p.m.
Ppt.
Supernatant, " P_{Mn} ", 12.6 ml.
Ppt.
Supernatant
6 ml. of phosphate buffer was added
33.2 ml. of $(NH_4)_2$-SO_4 sat. soln. was added, centrif. 10000 r.p.m.
" P_{33} ", 6.0 ml.
Ppt.
Supernatant, " S_{67} "
phosphate buffer was added
" P_{67} ", 18.5 ml.

As shown in this table, hydrogenase activity was present in both the precipitate and the supernatant of the high speed centrifugation and its specific activity was higher in the precipitate. The hydrogenase contained in the supernatant was lost nearly completely by manganese treatment. The insoluble precipitate formed by this treatment had considerable activity of hydrogenase, but could not be extracted with phosphate buffer.

Activity of thiosulfate reductase remained still in solution after treatment with manganese chloride, although a partial loss of activity was observed. By the fractionation with ammonium sulfate, it was concentrated mainly in the P_{67} fraction. On the other hand, cytochrome was uniformly distributed in all fractions.

As can be seen from the results, the precipitate of the high speed centrifugation (P) was found to have hydrogenase activity but little of thiosulfate reductase, and on the other hand, fraction P_{67} had no hydrogenase activity, but was rich in thiosulfate reductase. So it was intended to reconstruct the system for thiosulfate reduction with hydrogen, using these preparations and methyl viologen as an intermedate carrier.

TABLE I

Results of Fractionation with Ammonium Sulfate

Fractions	Proteins N mg.	Hydrogenase		Thiosulfate reductase	
		total	unit/N	total	unit/N
Extracts	70	2200	31	4200	60
S	53	1700	32	3900	74
P	4.5	710	58	48	11
S_{Mn}	23	0	0	1700	74
P_{Mn}	—	62	—	300	—
S_{33}	19	0	0	2000	110
P_{33}	34	12	4	110	32
S_{67}	6.0	0	30	30	5
P_{67}	13.6	2	0.2	1900	140

For the abbreviations of the fractions, see Diagram 1.

The results of the experiment, shown in Fig. 1, indicate clearly that both the preparations and methyl viologen were required for the reaction and that, when one of these fractions had been boiled, the reaction did not take place.

Fractionation with Acetone—Fractionation with acetone was carried out for the supernatant of manganese treatment. But in this case some-

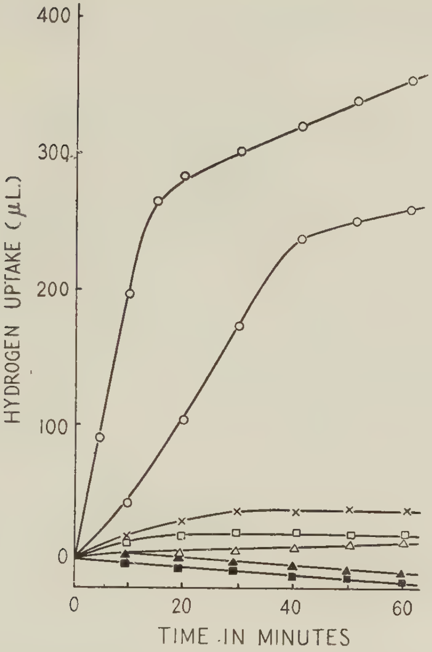


FIG. 1. Thiosulfate reduction with molecular hydrogen by the reconstructed system.

Reaction conditions: The main compartments of Warburg manometer vessels contained 1.0 ml. of Extracts (2.8 mg. N), or 1.0 ml. of Fraction P (0.42 mg. N) and 1.0 ml. of Fraction P_{67} (0.73 mg. N); the side arms, 0.2 ml. of methyl viologen (Mv) (0.04%) and 0.1 ml. of $Na_2S_2O_3$ (0.1 *M*); the center wells, 0.2 ml. of KOH (10%). Gas phase: H_2. Temperature: 30°. "Boiled" sample was prepared by heating on a water bath at 100°C for 5 minutes.

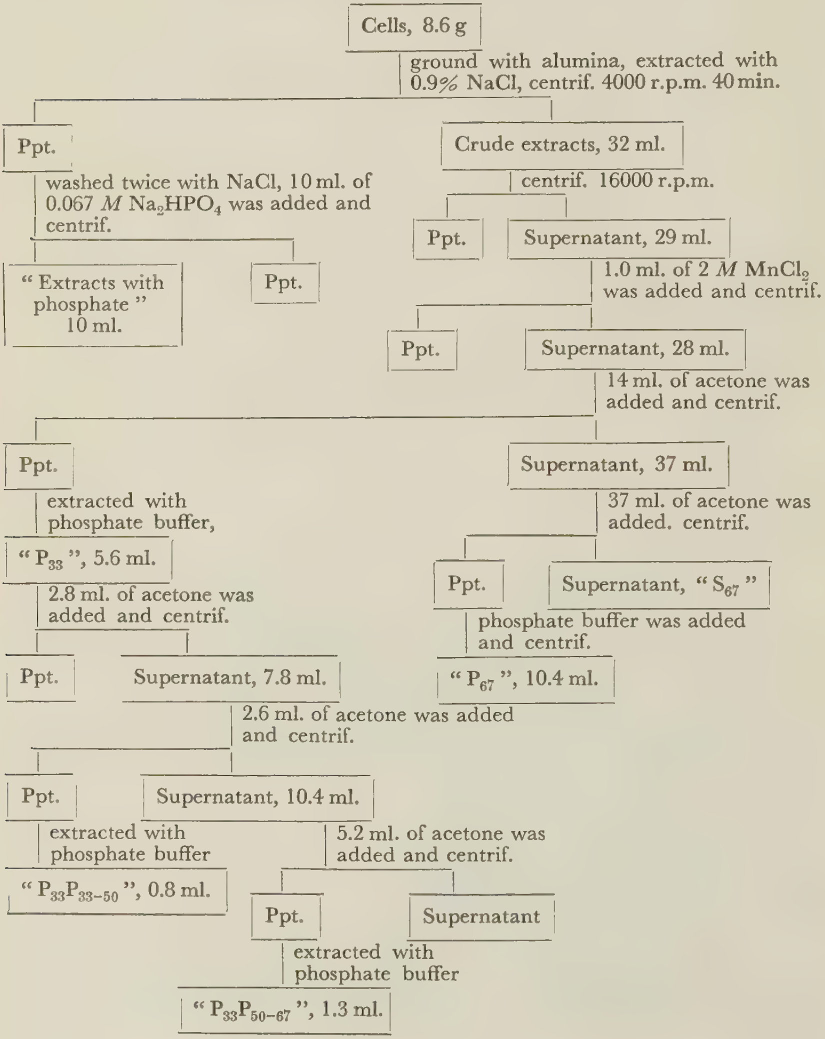
○ (above) Extracts$+$Mv$+S_2O_3^{--}$
○ $P+P_{67}+$Mv$+S_2O_3^{--}$
× $P+P_{67}+$Mv$+H_2O$
□ P+Boiled $P_{67}+$Mv$+S_2O_3^{--}$
△ Boiled $P+P_{67}+$Mv$+S_2O_3^{--}$
▲ $P+P_{67}+S_2O_3^{--}$
■ $P+S_2O_3^{--}$

When any components were omitted, water was added to make total volume to 2.3 ml.

what less amount (3/4) of manganese chloride was used. An example of the procedure is shown in Diagram 2.

DIAGRAM 2

Fractionation of Extracts of Sulfate-reducing Bacteria with Acetone


Cells, 8.6 g
ground with alumina, extracted with 0.9% NaCl, centrif. 4000 r.p.m. 40 min.
Ppt.
washed twice with NaCl, 10 ml. of 0.067 M Na₂HPO₄ was added and centrif.
" Extracts with phosphate " 10 ml.
Ppt.
Crude extracts, 32 ml.
centrif. 16000 r.p.m.
Ppt.
Supernatant, 29 ml.
1.0 ml. of 2 M MnCl₂ was added and centrif.
Ppt.
Supernatant, 28 ml.
14 ml. of acetone was added and centrif.
Ppt.
extracted with phosphate buffer,
" P33 ", 5.6 ml.
2.8 ml. of acetone was added and centrif.
Ppt.
Supernatant, 7.8 ml.
2.6 ml. of acetone was added and centrif.
Ppt.
extracted with phosphate buffer
" P33P33-50 ", 0.8 ml.
Supernatant, 10.4 ml.
5.2 ml. of acetone was added and centrif.
Ppt.
extracted with phosphate buffer
" P33P50-67 ", 1.3 ml.
Supernatant
Supernatant, 37 ml.
37 ml. of acetone was added. centrif.
Ppt.
phosphate buffer was added and centrif.
" P67 ", 10.4 ml.
Supernatant, " S67 "

The precipitates formed at 33 per cent (P_{33}) and between 33 per cent and 67 per cent (P_{67}) acetone were collected by centrifugation at low temperature. The precipitate at 33 per cent acetone (P_{33}) was fibrous and dark green and, when extracted with phosphate buffer, pH 7.0, green solution was obtained. This sample was fractionated again with acetone and its results were listed in Table II.

TABLE II

Results of Fractionation with Acetone

Fraction	Thiosulfate reductase		Cytochrome		Green pigment	
	total	unit/N	total	unit/N	total	unit/N
P_{33}	32	13	−0.06	−0.025	0.48	0.21
$P_{33}P_{33-50}$			0.015	0.050	0.015	0.05
$P_{33}P_{30-67}$			0.006	0.059	0.192	1.60
P_{67}	78	110	0.74	1.01	0.260	0.356
S_{67}			−0.14	—	0	0
Extracts with phosphate	34	8.5	1.93	0.48	−0.02	−0.005

For the abbreviations of the fractions, see Diagram 2.

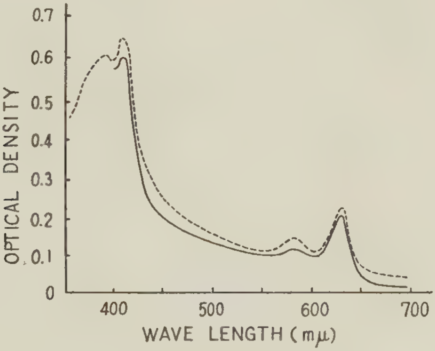


FIG. 2. Absorption spectra of the green pioment of the sulfate-reducing bacetria.

The fraction $P_{33}P_{67}$, the precipitate at 33 per cent acetone concentration, reprecipitated at 50 to 67 per cent acetone, was used. Light path : 1 cm. Contents of nitrogen : 0.04 mg./ml.

The precipitate separated from 50 to 67 per cent acetone from P_{33} (P_{33}-P_{67}) contained the greater part of the green pigment. Its absorption spectra are shown in Fig. 2.

Thiosulfate reductase was found chiefly in the P_{67} fraction. This fraction also contained the greater part of the cytochrome and a little amount of the green pigment. A cytochrome preparation, almost free from green pigment, was obtained by extraction with 0.067 *M* disodium phosphate from the residue of the ground cells with alumina after three times extractions with saline. This had only weak activity of thiosulfate reductase and hydrogenase, and contained scarcely any trace of the green pigment. The absorption spectra are shown in Fig. 3.

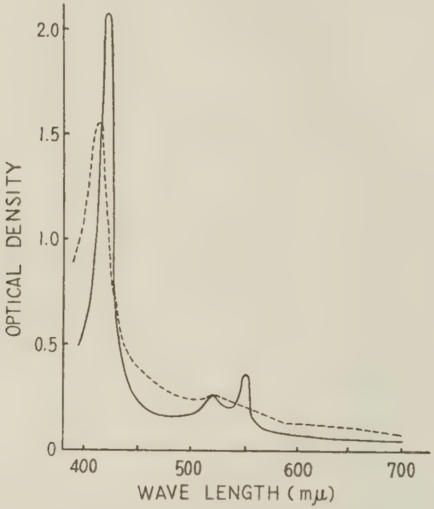


Fig. 3. Absorption spectra of the cytochrome of the sulfate-reducing bacteria.

"The extracts with phosphate" (Diagram 2) was used. Light path: 1 cm. Contents of nitrogen: 0.04 mg./ml. The broken line indicates the reduced form.

DISCUSSION

Molecular hydrogen can be activated by numerous microorganisms of widely different physological types, and, as a consequence, can serve as a reductant for reduction of diverse hydrogen acceptors. These reaction systems are complex, and it is believed that the reduction is carried out by specific reducing enzyme to each acceptor, coupling with hydrogenase presumed to be almost common to the numerous systems and responsible for the activation of molecular hydrogen.

Although, much progress has been made recently in the studies of hydrogenase, the properties of the hydrogen carrier systems, through which hydrogen activated by hydrogenase can be transferred to ultimate acceptors, have been known in only few cases.

The sulfate-reducing bacteria oxidize hydrogen with sulfate and also with more reduced inorganic sulfur compounds. In these reactions it was anticipated that various reducing enzymes linked with hydrogenase.

In the previous paper, from the experiments on thiosulfate reduction with hydrogen by cell-free extracts obtained from the bacteria, the following scheme was proposed ;

$$H_2 \underset{\text{hydrogenase}}{\cdots\cdots\cdots\cdots} \begin{array}{c}\text{Intermediary}\\ \text{carrier}\end{array} \underset{\begin{array}{c}\text{thiosulfate}\\ \text{reductase}\end{array}}{\cdots\cdots\cdots\cdots} S_2O_3^{--}$$

and some properties of thiosulfate reductase were studied.

In the experiments reported in the present paper, it was succeeded to separate hydrogenase and thiosulfate reductase from each other. The reconstruction study with these enzyme fractions disclosed that the reduction of thiosulfate to sulfite and sulfide with molecular hydrogen required hydrogenase and thiosulfate reductase and was mediated by methyl viologen. Also in living cells of the bacteria, the thiosulfate reductase and hydrogenase should play a role in thiosulfate reduction with molecular hydrogen.

The authors found previously (*1*), in confirmation of the report of Postgate (*4*), that the sulfate-reducing bacteria contained a cytochrome-like red pigment and a green one in spite of these strictly anaerobic charactor, and some properties of these pigments were studied, using the crude extracts.

The separation of the cytochrome and the green pigment made possible to confirm the positions of their absorption bands. This cytochrome had absorption maxima at 553, 525 and 419 mμ in the reduced form and 410 mμ in the oxidized. The preparations of the green pigment had absorption maxima at 630, 585, 410 and 390 mμ, which were not affected by addition of dithionite. Two maxima at the position of Soret band—a sharp maximum at 410 mμ and a second rather broad one at 390 mμ—were found in the all preparations obtained. Whether these preparations contain several pigments or this phenomenon is a charactor of one pigment is remained to be determined. But the relative height of these absorption peaks differed, though a little, in several preparations, so it may be possible that they contained several pigments.

The functions of these pigments in the living sulfate-reducing bacteria are as yet obscure. But it is anticipated that the cytochrome may be an intermediary hydrogen carrier involved in reducing systems for sulfate and other sulfur compounds. This view was indirectly supported by the observation that the cytochrome was reduced with hydrogen and formate, and oxidized with sulfate, sulfite and thiosulfate in the cell suspensions. But in this connection, further studies are necessary for this purpose.

SUMMARY

Extracts of sulfate-reducing bacteria were fractionated with ammonium sulfate or acetone and hydrogenase, thiosulfate reductase, cytochrome and green pigment were partially purified for the purpose of separation. Some properties of these enzymes and pigments are reported. Reconstruction of thiosulfate reducing systems was carried out successfully with hydrogenase and thiosulfate reductase preparations.

The authors wish to thank Dr. T. Soda and Dr. S. Akabori for their kind guidance and Dr. H. Tamiya for his valuable advices throughout this investigation. The expences for the work were aided partly by the Scientific Research Funds from the Ministry of Education.

REFERENCES

(*1*) Ishimoto, M., Koyama, J., and Nagai, Y., *J. Biochem.*, **41**, 763 (1954)
(*2*) Ishimoto, M., Koyama, J., and Nagai, Y., *J. Biochem.*, **42**, 41 (1955)
(*3*) Ishimoto, M., Koyama, J., Omura, T., and Nagai, Y., *J. Biochem.*, **41**, 537 (1954)
(*4*) Postgate, J., *Biochem. J.*, **56**, ix (1954)

The Journal of Biochemistry, Vol. 44, No. 4, 1957

BIOCHEMICAL STUDIES ON THE FRESH-WATER MEDUSA CRASPEDACUSTA SOWERBYI LANKESTER

I. ON THE INORGANIC COMPOSITION[1][2]

By YOSHIO OKUDA

(*Biological Institute of Kobe University, Kobe*)

(Received for publication, February 11, 1957)

After World War II, the fresh-water medusa, *Craspedacusta sowerbyi* Lankester, was reported to occur in rivers, ponds, swamps, and reservoirs in various areas of Japan, such as Hokkaido, Tohoku, Kanto, Kinki, and Kyushu (Uchida, 1955). Although this medusa has a world-wide distribution, it is mainly restricted to the warm regions of North America, Asia, and Europe. Moreover, it rarely settles in the same place. It is a very curious phenomenon that this fresh-water medusa, different from *C. iseana*, was almost unknown in Japan before World War II.

Up to the present time, morphological, embryological, and ecological studies of *C. sowerbyi* have been undertaken frequently, but there has been no precise biochemical information, except a report on its water content (Ohkawa, 1952).

The present paper contains the first report of a survey on the content of the principal inorganic elements of this fresh-water medusa.

On publishing the present report, the author wishes to thanks Dr. M. Tomita for his valuable advice and criticism.

MATERIALS AND METHODS

I discovered the fresh-water medusa *C. sowerbyi* at Itamiike in the vicinity of Itami City, in the south-eastern part of Hyogo prefecture. In 1954 this medusa appeared from the middle of September to the beginning of October. The next year it appeared in the middle of July and disappeared at the beginning of September. I took the opportunity to make a collection of these living animals, but only females could be obtained. These animals were carried to the laboratory and were first washed in distilled water, care being taken not to injure the body. Needless to say, during these operations, every possible precaution was taken to avoid contamination.

---

[1] Contributions from the Laboratory of Biological Institute, Kobe University, Kobe, No. 39.

[2] Part of this paper was read at the annual meeting of the Zoological Society of Japan, held at Fukuoka in October, 1955.

The medusae were desiccated at 105° in an electric-dryer. The dried specimen was powdered in an agate mortar, placed on carbon-electrodes and then burned by the arc-light, the arc-spectrum of which, coming through the slit of a quartz spectroscope and being focused by the quartz lens, was photographed.

The spectral analysing apparatus used here was of the Shimadzu type QF 60 and the spectrogram analysis was made through the combalater. Determination of the elements depended on Kayser's table. The quantity of each element was determined by chemical analysis. For comparison with a marine medusa, *Aurelia aurita* was collected from the seashore of Mikage and Suma of Osaka Bay. This material was treated by the same methods. The spectroscope was used as a method of detecting various elements in animal tissue, for it saves time and energy as compared with chemical analysis, thus supplying a valuable tool for making a wide survey of tissue contents.

RESULTS

The fresh material *C. sowerbyi* was weighed on a chemical balance after any attached water was removed, it was difficult to take away the attached water entirely, for the body is soft and fragile. Therefore the specimens, 150 in all, were weighed as wet bodies. The total wet weight of the medusae was 31.7358 g. In a completely desiccated condition, the weight was from 0.1714 g. to 0.5554 g. A calculation shows that the water-content averages from 98.25 per cent to 99.46 per cent of the fresh body. The results of these experiments are shown in Table I.

TABLE I

The Percentage of the Total Solids, Water and Ash of the Medusae, C. sowerbyi and A. aurita

	Total solids	Water	Ash
	%	%	%
Craspedacusta sowerbyi	1.10±0.65	98.86±0.60	0.11
Aurelia aurita	3.43±0.55	96.57±0.55	2.57

Elements were determined by their spectral lines, which corresponded to the following wave-lengths, *i.e.*, 4984, 4979, 4752, 4748, 4546, 4543, 3303, 2853, 2681, Å lines for sodium, 3440 Å line for potassium, 4878, 4586, 3181, 3159, 3007, 3001, 2997, 2995, Å lines for calcium, 3097, 3093, 3091, 2852, 2803, 2793, 2777, Å lines for magnesium, 4794, 4810, 4819, Å lines for chlorine, 2553.3, 2535.6, 2523, Å lines for phosphorus, 2529, 2519, 2516, 2514, 2507, Å lines for silicon, 2518.1, 2941.4, 3020.7, 3440.6, 3745.6, Å lines for iron, and 3274, 3247, 4063, 4023, Å lines for copper.

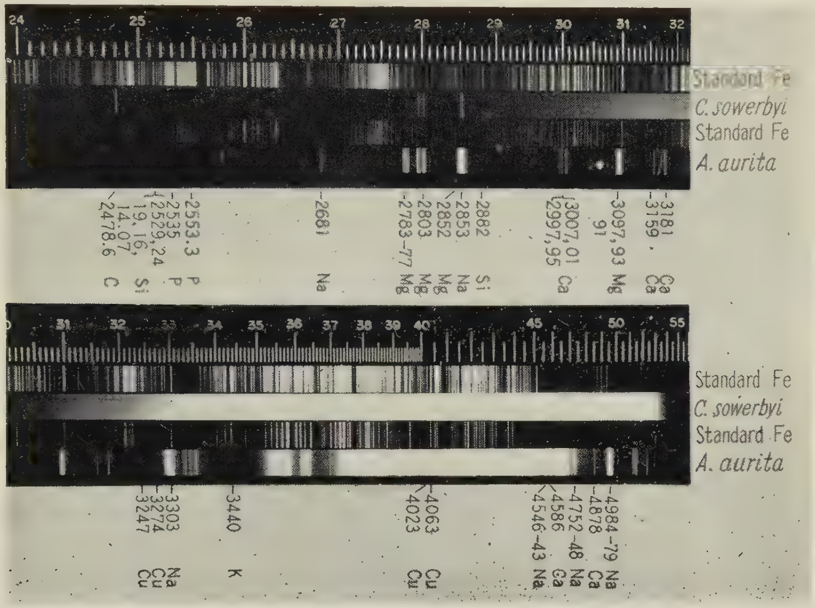


FIG. 1. The spectrogram of components of medusae, *C. sowerbyi* and *A. aurita*. (Scale reading ×100=Wavelength in Å.) The red-end is on the right of the lower part of the plate.

From 172.2261 g. of fresh medusae of *C. sowerbyi*, 1.9806 g. of completely desiccated material (1.15%) was obtained. In the case of *A. aurita*, 2.1566 g. of dried material was obtained from 33.7201 g. of fresh medusae by the same method. The results of quantitative analysis of this material are given in the Table II.

CONSIDERATION

The water content of the fresh-water medusa *C. sowerbyi* was calculated by Ohkawa (1952) to amount to from 97.5 per cent to 98.7 per cent of the body weight. The present author examined 150 speci-

Table II

The Principal Inorganic Components of C. sowerbyi and A. aurita are (for 1 g. of dried material):

	C. sowerbyi	*A. aurita*
	%	%
Na	0.026	0.259
K	0.012	0.016
Ca	0.006	0.024
Mg	—	0.022
Cl	0.047	0.482

mens and found the water content of 98.86±0.60 per cent. The difference probably arises, from the fact that it is difficult, in any case, to clear away the attached water. Accordingly I may fairly presume that the present values are valid.

The total solids of the fresh-water medusa *C. sowerbyi* average 1.15 per cent of the body weight, one-third of the value for the marine medusa *A. aurita;* the ash of the fresh-water species averages 0.11 per cent, or about one-twentieth of the value for the marine species.

The inorganic components of the marine medusa *A. aurita* rank in order of decreasing amount as follows: chlorine, sodium, magnesium, calcium, and potassium; they are approximately or actually those of sea water. In the fresh-water medusa *C. sowerbyi* they rank: chlorine, sodium, potassium, calcium, and magnesium; in river water they rank: chlorine, sodium, calcium, potassium, and magnesium. In relation to Table II, the percentage of the weight of each component contained in the fresh-water medusae are shown in Table III.

Table III

The Proportion of the Principal Inorganic Elements of C. sowerbyi

Na	K	Ca	Mg	Cl
2.58	1.23	0.56	—	4.68

The ratios of these elements on the basis of Na = 100:

Na	K	Ca	Mg	Cl
100	47.67	21.71	—	181.40

Twice as much potassium as calcium was contained in the fresh-water medusa, whereas not as much potassium as sodium and calcium is contained in the marine medusa or ocean water. In ocean and river, as a medium, the proportion is different, the sodium being always more than the potassium in ocean and river water, while in sea water magnesium greatly exceeds the calcium and potassium. The salinity of this fresh-water medusa is less than 3 per cent of that of ocean water thouoh it is more than 15 times of that of river water. The proportion between sodium and chlorine is approximate to the proportion between those found in ocean water. These proportions and ratios are the results of a gradual adjustment, brought about in the far past, between the tissue and its internal inorganic composition.

The distribution of iron and copper in living matter is already known (Warburg, 1921 and others). Keilin (1925, '26, '29), Elvehjem (1931), Fox, H. M. and Ramege, H. (1931) have shown by spectrographic analysis that iron and copper occur in various animal tissues. The spectrogram taken by the author shows the existence of iron and copper in the fresh-water medusa.

SUMMARY

1. The present paper is a biochemical study of the inorganic composition of the fresh-water medusa *Craspedacusta sowerbyi* Lankester, with special reference to spectrographic and chemical methods of analysis.
2. I have found, as the inorganic components in the fresh-water medusa, sodium, potassium, calcium, magnesium, chlorine, phosphorus, silicon, iron, copper which were also recognized in the marine medusa *Aurelia aurita.*
3. It is a notable fact that iron and copper are present in this animal.
4. The ratio of potassium to sodium in *C. sowerbyi* is much less than the ratio of these two elements in fresh water.
5. The inorganic endowment of *C. sowerbyi* was sodium 25.82 mg., potassium 12.30 mg., magnesium trace, calcium 5.56 mg., chlorine 46.78 mg. in 1 g. of dried material.
6. The salinity of this medusa is about 3 persent of that of ocean water though it is more than 15 times of that of river water.

REFERENCES

(1) Uchida, T., *Ann. Zool. Jap.*, **28**, 2 (1955)
(2) M. Ohkawa, *Zool. Mag.* (in Jap.), **61**, 1 (1952)
(3) Kayser, R., *Phys. Rev.*, **48**, 98 (1935)
(4) Kayser, R., Tabelle der Hauptlinien der Linienspektren aller Elemente,
(5) Maruzen. Kagaku binran.
(6) Pantin, C. F. A., *Biol. Rev.*, **6**, 459 (1931)
(7) Macallum, A. B., *Jour. Physiol.*, **29**, 213 (1903)
(8) Macallum, A. B., *Proc. Roy. Soc.*, B. **82**, 602 (1910)
(9) Macallum, A. B., *Physiol. Rev.*, **6**, 316 (1926)
(10) Fox, H. M., and Ramage, H., *Proc. Roy. Soc.*, B. **118**, 157 (1931)
(11) Elvehjem, C. A., *J. Biol. Chem.*, **90**, 111 (1931)
(12) Warburg, O., *Biochem. Z.*, **119**, 134 (1921)

NOTE

The Journal of Biochemistry, Vol. 44, No. 4, 1957

A SUPPLEMENTARY NOTE ON " BILE ACIDS AND STEROIDS. III "

(BROMINATION OF 7-KETOCHOLANIC ACID DERIVATIVE)

By KEN'ICHI TAKEDA and TAICHIRO KOMENO

(*From the Research Laboratory, Shionogi and Co., Ltd., Osaka*)

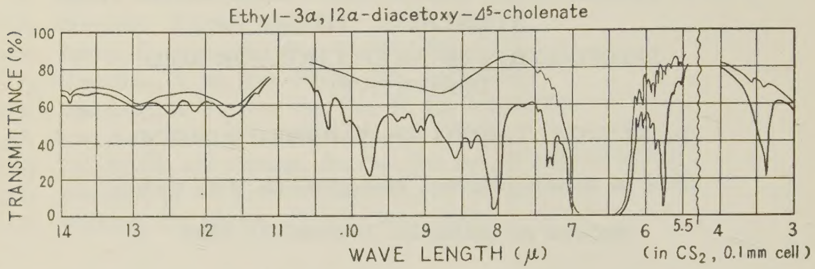
(Received for publication, December 17, 1956)

Previously the authors reported (*1*) that the bromination of ethyl 3 α, 12 α-diacetoxy-7-keto-cholanate in glacial acetic acid gave two kinds of bromides, m.p. 136° (II) and m.p. 162° (III). From the results of their optical rotations and the ultraviolet and infrared absorption spectra, it is difficult to assume that these bromides are the epimers of the bromine atom at C(6). Each bromide gave an analogous derivative having Δ^5-7-one group, m.p. 168° (IV) and m.p. 213° (V), respectively, by the dehydrobromination with pyridine and silver-nitrate. Furthermore, the melting point of each desoxo derivative, (VI) and (VII), prepared from the corresponding Δ^5-7-one type compound by the method of H. Hauptmann, showed almost the same value, m.p. 172° and 171°, and no depression was recognized on admixture. The analytical values of each series of compounds are all in agreement with the corresponding theoretical values. From these results we deduced, at that time, that these two bromides were the isomers of an unknown factor.

The results of the further investigation on these two bromides are showed by the following data.

The infrared spectrum of each desoxo derivative, (VI) and (VII), was examined thoroughly, and it was concluded that these two compounds were not identical with each other.

On the other hand, the melting point of the higher melting bromide, m.p. 162° (III), coincides comparatively with that of methyl 3α, 12α-diacetoxy-6α-bromo-7-ketocholanate (*2*), m.p. 165° (VIII). The mixed melting point of the synthesized methyl ester bromide (VIII) and the higher melting bromide (III) showed no depression and the infrared spectrum of each substance gave the identical results. This methyl ester bromide (VIII) also afforded methyl 3α, 12α-diacetoxy-Δ^5-7-keto-cholenate, m.p. 215° (IX), by the action of pyridine and silver-nitrate, which is identical with the above-mentioned Δ^5-7-one type compound


Ethyl-3α, 12α-diacetoxy-Δ^5-cholenate

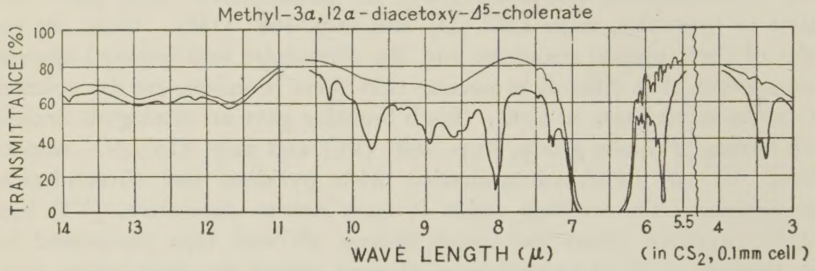

Methyl-3α, 12α-diacetoxy-Δ^5-cholenate

FIG. 1. I. R. spectra.

Ethyl 3α,12α-diacetoxy-7-ketocholanate (I) → 6α-Bromide m.p. 136° (II) → Δ^5-7-Keto-cholenate m.p. 168° (IV) → Ethyl Δ^5-cholenate m.p. 172° (VI)
Ethyl 3α,12α-diacetoxy-7-ketocholanate (I) → 6α-Bromide m.p. 162° (III) → Δ^5-7-Keto-cholenate m.p. 213° (V) → Δ^5-Cholenic acid derivative m.p. 171° (VII)
Methyl 3α,12α-diacetoxy-6α-bromo-7-ketocholanate m.p. 165° (VIII) → Methyl Δ^5-7-ketocholenate m.p. 215° (IX) → Methyl 3α,12α, diacetoxy-Δ^5-cholenate m.p. 171° (X)

(V). The Hauptmann's reduction of the foregoing Δ^5-7-one methyl ester (IX) gave methyl 3α, 12α-diacetoxy-Δ^5-cholenate, m.p. 171° (X), which is also identical with the substance, m.p. 171° (VII), derived from the higher melting bromide, as evidenced by its mixed melting point behavior, and the infrared absorption spectrum of (X) is in good agreement with that of (VII). In spite of this fact, no depression in melting point of this unsaturated methyl ester (X) was observed on admixture with ethyl 3α, 12α-diacetoxy-Δ^5-cholenate, m.p. 172° (VI).

From these experimental results, it is obvious that the above-mentioned two bromides are not the isomers but that they are different compounds which differ from each other only in the ester residue of the C(6)-α-bromo-acid: the lower melting bromide, m.p. 136° (II), is assigned as an anticipated ethyl 3α, 12α-diacetoxy-6α-bromo-7-oxo-cholanate and the higher melting one, m.p. 162° (III), is now confirmed as its methyl ester homologue. The transesterification might have occurred during the treatment of the impure bromo compound with methanol.

REFERENCES

(*1*) Takeda, K., and Komeno, T., *J. Biochem.*, **41**, 385 (1954)
(*2*) Corey, E. J., *J. Am. Chem. Soc.*, **76**, 175 (1954)